

The Role of TRIM39 in Cell Cycle and Apoptosis

by

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Dissertation submitted in partial fulfillment of
the requirements for the degree of Doctor
of Philosophy in the Department of
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ABSTRACT

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Abstract

Within individual cells, the opposing processes of proliferation and apoptosis are precisely regulated. When this regulatory balance is interrupted, cells may become abnormal or even transformed. Understanding how to reverse or avoid these detrimental transformative processes begins with an intimate knowledge of the processes governing the cell cycle and apoptosis. Cell proliferation is governed by the cell cycle machinery. The cell cycle is driven by Cyclin-dependent kinase (Cdk) activity, which is dependent on the availability of specific Cyclin binding partners. The amount of available Cyclin is tightly controlled by a ubiquitin ligase protein complex called the anaphase promoting complex/cyclosome (APC/C.) This complex mediates the timely ubiquitylation and degradation of cell cycle regulators in order to control mitotic exit, the G1/S transition and to respond to signals emanating from spindle assembly checkpoint.

Given the importance of the APC/C, cells develop many ways to regulate APC/C activity. Post-translational modifications of the APC/C have been shown to alter its functionality, and many pseudosubstrate-based inhibitors have been discovered. Moreover, inhibitors such as Emi1 and Emi2, have been showed to inhibit the APC/C through their own intrinsic ubiquitin E3 ligase activities. Utilizing the *Xenopus* egg extract system, our laboratory has previously demonstrated that the RING domain-

containing ubiquitin E3 ligase Xnf7 can inhibit *Xenopus* APC/C activity. In the thesis, we have identified TRIM39 as an Xnf7-related human regulator of the APC/C. Our study showed that TRIM39 restrains the ability of the APC/C to ubiquitylate Cyclin B in vitro and attenuates the degradation of Cyclin B and geminin when TRIM39 is incubated in cell lysates. Notably, it has been reported that TRIM39 activity is responsible for the accumulation of the Bax-interacting protein (and activator) MOAP-1 following etoposide-induced DNA damage. Our data indicated that MOAP-1 is a novel APC/C substrate, and that the ligase activity of TRIM39 appears to be essential for preventing its degradation. We further demonstrated that decreased levels of the APC/C activator Cdh1 induces MOAP-1 protein accumulation, thereby promoting DNA damage-induced apoptosis in 293T, PC3 and H1299 cells. This study illustrates a potential function for the APC/C in DNA damage induced apoptosis and also demonstrates that TRIM39 regulates both the cell cycle and apoptosis via APC/C inhibition.

To extend our observations regarding the role for TRIM39 in APC/C regulation, we investigated effects on the cell cycle via real-time imaging microscopy. We found cells arrest at G1/S in TRIM39 depleted RPE cells, a cell line which is commonly used for cell cycle analysis. This arrest phenotype is not observed in 293T, PC3 and H1299 cells which bear mutant p53 alleles. Further analysis showed that TRIM39 depleted RPE cells upregulate many genes that function downstream of p53 activity, such as the cdk inhibitor p21 — thus, arresting cells at G1/S and reducing proliferation. The reduced

growth can be rescued by p53 knockdown. Mechanistically, TRIM39 interacts with p53 and promotes destruction of p53 by ubiquitylation. This ubiquitylation is independent of the activity of the most intensively studied p53-directed E3 ligase, MDM2; depletion of both MDM2 and TRIM39 has a synergistic effect on p53 accumulation. This elevated p53 leads to more apoptosis in cancer cells bearing wildtype p53. Consequently, TRIM39 depletion might be employed as a combination treatment with MDM2 inhibitor, such as nutlin-3a, to stimulate tumor cell death.

In the thesis, we have found TRIM39 inhibits both the APC/C and p53. Both are essential regulators of cell cycle and apoptosis. Moreover, we have determined that the inhibitory activity of TRIM39 requires its E3 ligase activity. Future experiments will be directed towards investigating how TRIM39 protein stability and ligase activity are regulated to understand more fully the physiological situations in which TRIM39 is able to exert its ability to modulate the cell cycle and apoptosis. I will also discuss some preliminary data regarding changes in TRIM39 ligase activity induced by Chk1 and changes in TRIM39 protein abundance regulated by polo-like kinase 1(Plk1). Chk1 and Plk1 are essential kinases for cell cycle checkpoint and progression. Connecting Chk1 and Plk1 to TRIM39 may provide a more thorough understanding of TRIM39's ability to control the APC/C inhibition and p53 ubiquitylation in response to cell cycle or cell damage cues. Since the APC/C and p53 both can regulate cell cycle and apoptosis, further investigations into the involvement of TRIM39 in the life-or-death decision will

be of great interest.

Dedication

This work is dedicated to my family for their ceaseless support and encouragement; and to Ying-Cing for his trust, care and love.

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List of Abbreviations

APC/C	Anaphase promoting complex/cyclosome
Bcl-2	B cell lymphoma 2
β -Trcp	β -transducin repeat containing protein
BH	Bcl-2 homology
BUBR1	Budding uninhibited by benzimidazole
Caspase	Cysteine dependent aspartate-directed proteases
Cdk	Cyclin-dependent kinase
CHX	Cycloheximide
Cip/Kip	CDK interacting protein/Kinase inhibitory protein
CKIs	Cdk inhibitor proteins
Ctr	Control
CRD	C-terminal regulatory domain
CUEDC2	CUE domain-containing protein 2
D-box	Destruction box
DD	Death domain
DED	Death effector domain
DISC	Death inducing signaling complex
DSB	Double strand break

DSP	Dithiobis[succinimidyl propionate]
Edu	5-ethynyl-2-deoxyuridine
Emi1	Early mitotic inhibitor 1
FADD	Fas-associated death domain protein
FBW7	F-box/WD repeat-containing protein 7
G1	Gap1
G2	Gap2
HECT	Homologous to the E6-AP Carboxyl Terminus
hTERT-RPE	Human telomerase reverse transcriptase-immortalized retinal pigment epithelial cell line
INK4a/ARF	Inhibition of Kinase 4a/ Alternative Reading Frame
IP	Immunoprecipitation
M	Mitosis
MAD1	Mitotic arrest deficient 1
MBP	Maltose binding protein
MDM2	Murine double minute 2
MOAP-1	Modulator of apoptosis 1
MOMP	Mitochondria outer membrane permeabilization
Msl2	Male-specific lethal-2

MT	Mutant
NHEJ	Non-homologous end joining
PFA	Paraformaldehyde
PFKFB3	6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase isoform 3
PIDD	p53-Induced protein with Death Domain
PKGI	cGMP-dependent protein kinase I
Plk1	Polo-like kinase 1
PRD	Proline-rich domain
	RIPAssociated ICH-1/CED3 homologous protein with Death
RAIDD	Domain
RASSF1A	Ras association domain-containing family isoform A
Rb	Retinoblastoma protein
RING	Really Interesting New Gene
RRM2	Ribonucleotide reductase
S	Synthesis
SAC	Spindle assembly checkpoint
SCF	Skp1-Cullin-F-box
Skp1	S phase kinase-associated protein 1
SNP	Single nucleotide polymorphism

SSB	Single strand break
TAD	Transactivation domain
t-Bid	Cleaved Bid
TetraD	Tetramerization domain
TNF- α	Tumor necrosis factor α
TPRs	Tetratricopeptide repeats
TRADD	Tumor necrosis factor receptor type 1-associated DEATH domain
TRAIL	TNF related apoptosis inducing ligand
TRIM	Tripartite Motif
U/Ub	Ubiquitin
UPS	Ubiquitin-proteasome system
UV	Ultraviolet radiation
WT	Wildtype
WWP1	WW domain-containing protein 1
Xnf7	<i>Xenopus</i> nuclear factor 7

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1. Introduction

1.1 *Cell cycle progression*

Cell division is achieved by a series of ordered events, including DNA replication, spindle formation, chromosome segregation, cytokinesis and entry into the next division cycle. The cell cycle can be divided into four stages: G1 (Gap1), S (synthesis), G2 (Gap2) and M (mitosis) stage (Fig. 1.1). Through G1 to S phase, cells grow and produce their complement of proteins and nucleotides to prepare for accurate DNA replication in S phase. After DNA duplication, cells keep growing, and in the G2 phase, sister chromatids are connected in a cohesion-dependent protein complex. Duplicated chromosomes and organelles are then separated into two daughter cells during mitosis. Mitosis can be further subdivided into five sequential phases, known as prophase, prometaphase, metaphase, anaphase, and telophase. Prophase is a stage when the chromosomes become condensed and is followed by prometaphase when the nuclear membrane breaks down and mitotic spindle fibers attach at the kinetochore. Metaphase is reached when the spindle structure has properly formed and all of the chromosomes are aligned in preparation for sister chromatid segregation. This process is tightly regulated by the spindle assembly checkpoint (SAC) to ensure chromosomes attach to spindles properly. During anaphase, the SAC is inactivated to trigger proteolytic cleavage of cohesin, causing sister-chromatids to separate to the two

opposing poles of the cell, pulled by the spindle microtubules. The chromosomes are then decondensed and the nuclear membrane reforms in telophase. After M phase, cytokinesis begins. In this process, the cytoplasm and other cellular contents divide into the two daughter cells, completing cell division, and leading to G1 phase entry (Morgan, 2007; Viallard et al., 2001).

When there is a lack of sufficient growth factors and nutrients, or when cells are fully differentiated, such as neurons, cells can also exit G1 into a quiescent G0 phase through a cell cycle checkpoint called the restriction point. Cells in G0 phase can reenter G1 and continue to divide through cell cycle progression when growth factors stimulate cells. In contrast, another cellular state, called senescence, occurs when cells stop division and cannot re-enter the cell cycle. Senescent cells are characterized by their flat and enlarged shape; increased expression of β -galactosidase and Cyclin-dependent kinase inhibitors; and a transcriptionally inactive heterochromatic structure (Kuilman et al., 2010; Sherwood et al., 1988). Senescence plays a role in tumor suppression since it can suppress cell proliferation. It is also related to aging, as accumulation of senescent cells leads to impaired tissue homeostasis and eventually induce aging. (Collado et al., 2005; Sharpless, 2004)

1.1.1 Cdk kinase activity

Cyclin-dependent kinase (Cdk) activity controls progression of the cell cycle, and the activity of each kinase depends on the availability of its Cyclin partner. Cyclins D1, D2 and D3 pair with the G1 Cdk4/6. These complexes phosphorylate retinoblastoma protein (Rb), leading to its dissociation from and activation of the transcription factor E2F. E2F transcriptional targets, including Cyclin E1 and E2, are required for the G1/S transition. Cyclin E/Cdk2 promotes the G1/S transition and Cyclin A2 synthesis. Cyclin A2 then pairs with Cdk2 to enter S phase and trigger DNA and centrosome replication. When cells are ready, Cyclins B1 and B2 bind to Cdk1 and, together with CyclinA2/Cdk1, drive entry into mitosis. Activation of the CyclinA,B/Cdk1 complexes initiates prophase and leads to nuclear membrane breakdown. When Cyclin B/Cdk1 loses its activity, cells exit mitosis for G1 (Draetta and Beach, 1988; Morgan, 2007).

Cdk activity can also be regulated by Cdk inhibitor proteins (CKIs) in G1 phase. These CKIs are potent inhibitors of Cyclin/cdk complexes and are activated when cells face an unfavorable environment or DNA damage. There are two families of CKIs, the Cip/Kip family (CDK interacting protein/Kinase inhibitory protein) and the INK4a/ARF family (Inhibitor of Kinase4a/ Alternative Reading Frame). The Cip/Kip family includes p21, p27 and p57. These proteins can arrest cells at G1 both by inhibiting Cyclin E, A/Cdk2 and by activating the G1 Cyclin D/Cdk complex via binding to the Cyclin/Cdk

complexes. The INK4a/ARF family, including p15, p16, p18 and p19, can inhibit Cdk4 and Cdk6 activity by binding to Cdk4 or Cdk6 (Sanchez and Dynlacht, 2005) (Morgan, 2007; Viallard et al., 2001) (Sherr and Roberts, 1999).

1.1.2 Ubiquitin ligase in the cell cycle

Timely regulation of cell cycle progression is mainly achieved by controlling the abundance of key regulatory proteins. Waves of protein expression levels are established with coordinated degradation by the ubiquitin-proteasome system (UPS) (Ciechanover, 1998). Ubiquitylation, a post-translational modification process in which ubiquitin peptides are conjugated to Lys residues of cell regulatory proteins, has a major influence on protein activity either via proteasome-mediated degradation or non-proteolytic regulation. Target substrates are processed sequentially by a series of ubiquitin enzymes: an ubiquitin E1 activating enzyme, an E2 conjugating enzyme and an E3 ligase (Fig. 1.2) (Budhidarmo et al., 2012; Wasch and Engelbert, 2005). The specificity of substrate determination is controlled largely by E3 ligases. There are nearly 600 E3 ligases in humans and many E3 ligases harbor a Really Interesting New Gene (RING) domain or a Homologous to the E6-AP Carboxyl Terminus (HECT) domain. The RING domain is a conserved motif, often containing Cys-X2-Cys-X11-16-Cys-X-His-X2-Cys-X2-Cys-X7-74-Cys-X2-Cys (C3HC4), where X can be any amino acid. There are several C3HC4 type RING-containing E3 ligase proteins, including Cbl, BRCA1, and

murine double minute 2 (MDM2). Another type of RING domain, in which Cys5 is substituted with His (H2 type), is found in Rbx1 and APC11 proteins. These two proteins are subunits of the Skp1-Cullin-F-box (SCF) and Anaphase promoting complex/cyclosome (APC/C) complexes, respectively (Lipkowitz and Weissman, 2011) (Ikeda and Inoue, 2012) (Benanti, 2012).

SCF and APC/C are the most studied and characterized E3 ligases involved in controlling cell cycle progression. SCF is composed of: a structural subunit, cullin; a RING domain containing protein Rbx1; an adaptor protein S phase kinase-associated protein 1 (Skp1); and one of up-to 69 human F-box proteins, which serves to recognize specific substrates. Therefore, the activity of SCF toward its substrates is modulated by the availability of F-box proteins. In most F-box proteins, including F-box/WD repeat-containing protein 7 (FBW7) and β -transducin repeat containing protein (β -TrCP), the F-box domain interacts with phosphorylated substrates. This interaction regulates SCF activity. Although SCF E3 ligases are active throughout the cell cycle, most SCF substrates are targeted in G1 and S phase. SCF (including SCF^{Fbx4- α BCrystallin}, SCF^{Fbxw8}, SCF ^{β -Trcp}, SCF^{Fbxo31}, and SCF^{Skp2}) can ubiquitylate Cyclin D1, and SCF^{Fbw7} is reported to ubiquitylate phosphorylated Cyclin E and Myc (Barbash and Diehl, 2008) (Nakayama et al., 2001). Moreover, SCF^{Skp2} targets Cyclin dependent kinase inhibitors p21, p27 and p57

(Lipkowitz and Weissman, 2011; Nakayama and Nakayama, 2006) (Benanti, 2012) (Cardozo and Pagano, 2004).

The APC/C also contains a RING domain protein and is active during mitosis and G1 phase. Its co-activators, Cdc20 and Cdh1, associate with the APC/C at mitosis and G1, respectively, and mediate substrate recruitment. The APC/C is activated in mitosis to degrade Cyclin A in prophase, followed by Cyclin B and the separase inhibitor, securin, to transit the cycle from metaphase to anaphase. The APC/C is activated in G1 to prevent premature entry into S phase through the degradation of Cyclins and proteins regulating replication, such as geminin. Other cell cycle regulators ubiquitinated by the APC/C include polo-like kinase 1 (Plk1), p21 and Aurora B (Shirayama et al., 1999) (Geley et al., 2001) (Lindon and Pines, 2004) (Amador et al., 2007) (Nguyen et al., 2005). Interestingly, crosstalk between the APC/C and SCF occurs. SCF promotes the activation of the APC/C by degrading its inhibitor, Early mitotic inhibitor 1 (Emi1), during interphase, while Skp2 is targeted by APC/C leading to SCF inactivation (Bashir et al., 2004) (Moshe et al., 2004) (Cardozo and Pagano, 2004). Thus, these two RING-containing ligases work closely together to regulate and promote cell cycle progression.

1.2 DNA damage response

The human genome is under constant threat from both exogenous sources, such as ionizing radiation, ultraviolet radiation (UV), and alkylating chemicals, and endogenous sources, such as reactive oxygen species, nitrogen species, and DNA replication errors (Lindahl, 1993). Given the importance of maintaining the integrity of the genome, cells have developed robust mechanisms to overcome these damages. These mechanisms include sensing DNA damage, using cell cycle check points, and repairing DNA. In the case of severe damage, cells will activate a death pathway, such as apoptosis (Jackson and Bartek, 2009).

1.2.1 DNA damage sensing signaling and cell cycle check point

DNA damage sensors detect lesions of DNA damage and initiate the DNA damage response. The key sensors are the protein kinases ATM and ATR, which are recruited and activated by double strand breaks (DSBs) or single strand breaks (SSBs) (Jackson and Bartek, 2009). ATM is recruited by the MRN complex (Mre11- Rad50- Nbs1), and will dissociate from inactive dimers and undergo autophosphorylation after DSBs and SSBs. Upon sensing SSBs, ATR is recruited by RPA and ATRIP, and PCNA-related 911 (Rad9-Rad1-Hus1) is recruited to the complex with DNA (Harper and Elledge, 2007) (Lee and Paull, 2005). ATM and ATR propagate the signal by

phosphorylating many substrates including Chk1 and Chk2 kinases, which play a key role in inducing cell cycle arrest and DNA repair.

Chk1/2 go on to phosphorylate Cdc25A, a phosphatase that targets Cdk. This phosphorylation creates a docking site for SCF ^{β -Trcp} and consequently Cdc25A ubiquitylation and degradation. Destruction of Cdc25A interferes with progression of S phase due to the accumulation of inhibitory phosphorylations on Cdk, thereby leading to the intra-S checkpoint. Phosphorylation of Cdc25C by Chk1/2 sequesters 14-3-3 and inhibits Cdc25C dephosphorylation of Cdk. Together, this inhibits entry into mitosis (G2/M checkpoint) (Harper and Elledge, 2007) (Guardavaccaro and Pagano, 2006). In addition, Chk1/2 can phosphorylate Rb to promote its inhibitory binding to E2F. Chk1/2 can also phosphorylate p53 to prevent its degradation and to promote its transcriptional activity. The two phosphorylations on Rb and p53 result in G1/S arrest. Taken together, Chk1/2 ensure that cells arrest at G1/S, intra S, or G2 during the DNA damage response. This arrest is necessary to provide cells with time to repair their damaged DNA (Inoue et al., 2007) (Nowsheen and Yang, 2012).

1.2.2 Check point recovery and adaptation

During G2/M arrest, polo-like kinase 1 (Plk1) is inhibited by ATM/ATR signaling, preventing Plk1 from phosphorylating and inhibiting wee1, a kinase that in turn mediates an inhibitory phosphorylation of Cdk. Specifically, this Plk1

phosphorylation of wee1 facilitates wee1 ubiquitylation, so Plk1 inhibition causes wee1 accumulation, robust Cdk inhibition, and G2/M arrest. Plk1 inhibition also prevents activation of the APC/C since APC/C activation requires phosphorylation of its subunits by Plk1 (Tsvetkov and Stern, 2005) (Guardavaccaro and Pagano, 2006) (Smits et al., 2000).

Plk1 is reported to be an activating kinase for Cdc25, although this function is dispensable in unstressed cells. However, this function is important for recovery from G2/M arrest. Phosphorylation of Cdc25B by Plk1 is required for mitotic entry after DNA damage (van Vugt et al., 2004). Plk1 is also involved in the inactivation of Chk1 in that it phosphorylates Claspin, an activator of Chk1, targeting it for SCF ^{β -Trcp}-mediated ubiquitylation and destruction (Gewurz and Harper, 2006). Plk1 was also shown to phosphorylate 53BP1 and Chk2 to inactivate G2 arrest and promote re-entry into the cell cycle (van Vugt et al., 2010). Furthermore, Plk1 can phosphorylate a p53 negative regulator, GTSE1, which can promote p53 shuttling out of the nucleus and degradation (Bahassi et al., 2011) (Liu et al., 2010).

It has been reported in yeast, *Xenopus*, and human cancer cell lines that cell division will occur even in the presence of unrepaired DNA after a prolonged G2 arrest. This process is called checkpoint adaptation. In yeast, checkpoint adaptation requires the activity of polo-like kinase, Cdc5, CKII, phosphatases Ptc2 and Ptc3, and inactivation

of Rad53 (Chk2 homolog) and Chk1. Recent data also showed that overexpression of Cdc5 will override DSB-induced signaling (Bahassi et al., 2011). In *Xenopus*, Plk1 phosphorylates Claspin, and this phosphorylation reduces the association between Claspin and chromatin, leading to Chk1 inactivation (Lupardus and Cimprich, 2004). In human cells, it has been suggested that Chk1 inhibition and Plk1 kinase activation are required for adaptation. It has been postulated that adaptation could move the cell into another cell cycle phase for cell death (mitotic catastrophe) or DNA repair (G1/S arrest) in different stages of arrest. However, apoptosis should be a safer and more efficient strategy than checkpoint adaptation for preventing the transmission of damage to future generations. Moreover, checkpoint adaptation is proposed to play a role in cancer development by allowing the propagation of oncogenic genomic aberrations (Syljuasen et al., 2006). Interestingly, high Plk1 expression has been reported in many types of cancer (Takai et al., 2005). This elevation may provide evidence correlating checkpoint adaptation and cancer development. Figure 1.3 summarizes the DNA damage response described above.

1.3 APC/C

1.3.1 The role of APC/C

The APC/C is a large multisubunit E3 ubiquitin ligase that targets substrate degradation through the 26S proteasome. Temporal regulation of APC/C activity and

substrate specificity is critical for APC/C-mediated coordination of cell cycle progression. As described earlier, the APC/C degrades many essential regulators in mitosis and accurately controls chromosome segregation to prevent chromosome instability. Besides its role in mitotic exit, the APC/C^{Cdh1} is involved in maintaining low Cdk1 activity in G1 by degrading Cyclin A, Cyclin B, Cdc25A and Skp2. APC/C^{Cdh1} also regulates DNA replication by controlling the degradation of geminin and cdc6, which regulate replication and pre-replication complex formation (Manchado et al., 2010) (Fig. 1.5).

Aside from its roles in cell cycle progression, the APC/C has been reported to control programmed cell death and neural growth. The APC/C^{Cdh1} is highly expressed in the brain. It inhibits axon growth by degrading the transcription co-repressor SnoN and Id2 (Konishi et al., 2004; Lasorella et al., 2006; Stegmuller et al., 2006). Moreover, Cdh1 depletion in neuroblastoma and post-mitotic neurons leads to Cyclin B accumulation and apoptosis. This apoptosis is suggested to result from cell cycle re-entry when Cyclin B levels are elevated. Such elevated Cyclin B is found in degenerating brains with Alzheimer's disease or stroke (Almeida et al., 2005). Another study substantiates the role of Cdh1 in attenuation of neuronal apoptosis (Maestre et al., 2008). Stimulating the N-methyl-D-aspartate receptors in such a way as to mimic

neurodegenerative diseases leads to Cdk5 mediated-phosphorylation and inhibition of Cdh1. This inhibition results in Cyclin B accumulation and apoptosis.

The APC/C is also involved in metabolic regulation: it is reported to ubiquitylate glycolysis-promoting enzyme 6-phosphofructo-2-kinase/fructose-2, 6-bisphosphatase isoform 3 (PFKFB3) in T lymphocytes and cortical neuron cells (Colombo et al., 2010; Rodriguez-Rodriguez et al., 2012). The APC/C can also affect glutaminolysis by targeting glutaminase-1. Moreover, the APC/C can regulate nucleotide biosynthesis by targeting thymidine kinase 1 and ribonucleotide reductase 2 (Manchado et al., 2010).

1.3.2 APC/C structure

The APC/C is composed of at least fifteen subunits, and the recognition of substrates is mediated by one of its co-activators, Cdh1 or Cdc20, during the mitotic cell cycle, whereas Ama1 acts in yeast meiosis (Dong et al., 1997) (Schwab et al., 1997) (Cooper et al., 2000) (Fig. 1.4). APC/C subunits are held together by APC1, the largest of the subunits. The cullin-domain of APC2 is associated with the RING-finger domain of APC11 to form the catalytic subcomplex, which catalyzes the direct transfer of a ubiquitin moiety from the E2 to its substrates (Pines, 2011). The majority of the other subunits, such as APC3/Cdc27, APC5, APC6 and APC7, contain a series of tetratricopeptide repeats (TPRs) that mediate interdependent associations. TPRs are also important for binding to Cdh1 and Cdc20 (Vodermaier et al., 2003). The co-activators

use three motifs to interact with the APC/C. These are the C box, KILR (Lys-Iso-Leu-Arg) motif, and IR (Iso-Arg) motifs. After associating with the APC/C, these co-activators help the APC/C recognize its substrates through an interaction with the substrate's consensus motif (e.g., D-box: RXXL or KEN box) (Kraft et al., 2005) (Pfleger and Kirschner, 2000). While both APC/C^{Cdc20} and APC/C^{Cdh1} recognize the D-box, only APC/C^{Cdh1} recognizes the KEN box. APC10/Doc1 has also been reported to enhance the APC/C's substrate binding by forming a substrate recognition co-receptor with Cdh1 (Buschhorn et al., 2011). In addition, the IR tail of Cdh1 and APC10 both engage with APC3. The co-activator binds to the TPR of APC3, and this interaction both facilitates substrate recruitment to the APC/C and enhances the specificity of the APC/C (Peters, 2006) (Thornton et al., 2006) (Matyskiela and Morgan, 2009) (da Fonseca et al., 2011). For the APC/C to catalyze substrate ubiquitylation, an E2, either Ubc5 (including Ubc5a, Ubc5b and Ubc5c) or UbcH10, is required for initiation, while another E2, UBE2S, assembles extended K11-linked polyubiquitin chains on substrates (Wu et al., 2010) (Garnett et al., 2009). Mutants of UbcH10 can recapitulate the loss of APC/C activity, but UbcH5 mutants cannot. Moreover, depletion of UBE2S has been shown to have a more significant effect on ubiquitin chain elongation of APC/C targets than depletion of UbcH10 (Peters, 2006) (Wu et al., 2010) (Garnett et al., 2009).

1.3.3 Regulation of the APC/C

The APC/C activity is tightly controlled via phosphorylation-regulated co-activator binding, auto-destruction of Cdh1, UbcH10, and binding of inhibitory proteins (Fig. 1.6).

1.3.3.1 Regulation of co-activators

Phosphorylation of APC/C subunits by mitotic kinases like Cyclin B/Cdk1 and Plk1 increases the affinity of Cdc20 for the core APC/C at M phase. In contrast, phosphorylation of Cdh1 by S, G2 and M-Cdks inhibits its association with the APC/C (Kraft et al., 2003) (Eckerdt and Strebhardt, 2006). During M phase, APC/C^{Cdc20} targets Cyclin B for degradation leading to reduced mitotic Cdk activity; phosphatases subsequently increase its dephosphorylation activity. This temporally -regulated event allows Cdh1 dephosphorylation and APC/C^{Cdh1} activation during mitotic exit. In turn, APC/C^{Cdh1} marks Cdc20 for destruction, consequently preventing the two co-activators from activating APC/C at the same time. Phosphorylation of Cdh1 can also sequester Cdh1 to the cytoplasm from the nucleus, and phosphorylated Cdh1 can be targeted by SCF for degradation. Therefore, phosphorylation of Cdh1 by Cdks initiates an efficient inactivation of the APC/C (Peters, 2006).

1.3.3.2 Self-destruction of Cdh1 and UbcH10

In addition to SCF, Cdh1 has been reported to be ubiquitinated by APC/C^{Cdh1} itself. The autoregulation of Cdh1 in a feedback loop maintains its lower protein level when active, and thus is important for timely inactivation of the APC/C. UbcH10 can also trigger its own destruction when there are not many substrates of the APC/C at the G1/S transition. This auto-ubiquitylation will attenuate APC/C activity, letting Cyclin A accumulate and activate Cdk, which in turn phosphorylates Cdh1 to completely inhibit the APC/C (Rape and Kirschner, 2004).

1.3.3.3 APC/C inhibitors

Given the pivotal role of timely regulation of the APC/C, there are different protein inhibitors acting at different cell cycle stages (Table 1). Emi1 is expressed at the G1/S transition and inhibits the APC/C by competing with its substrates for binding, suppressing ubiquitin chain elongation, inhibiting ubiquitin transfer, and interrupting E2-APC/C association from S to G2 phase. The inhibition of the APC/C by Emi1 prevents geminin accumulation and DNA re-replication. When cells enter M phase, Emi1 is degraded by SCF ^{β -TrCP} (Wang and Kirschner, 2013) (Di Fiore and Pines, 2007) (Machida and Dutta, 2007).

After cells enter mitosis, Cdk phosphorylates the APC/C and promotes APC/C^{Cdc20} assembly to degrade Cyclin A and Nek2A. However, the APC/C is prevented

from degrading Cyclin B and securin by the SAC. SAC proteins, including mitotic arrest deficient 1 (MAD1), MAD2, budding uninhibited by benzimidazole (BUB)R1, BUB3 and MPS1, are recruited to unattached kinetochores to generate the mitotic checkpoint protein complex (MCC) with Cdc20 and to inhibit Cdc20 (Fang, 2002) (Eytan et al., 2013). The inhibition is partly because BUBR1 can act as a pseudosubstrate inhibitor of Cdc20, and also because BUB1 mediates an inactivating phosphorylation on Cdc20 (Burton and Solomon, 2007) (Tang et al., 2004). Until the SAC is satisfied (i.e. the chromosomes are aligned properly), Cdc20 is auto-ubiquitylated by APC/C and released from the MCC, which is dependent on APC15 and APC11 (Mansfeld et al., 2011) (Foster and Morgan, 2012).

In addition to these SAC proteins, it has been reported that Ras association domain-containing family isoform A (RASSF1A) and RAE-NUP98 can inhibit the early mitotic APC/C. RASSF1A is suggested to interact with Cdc20 at prometaphase after Emi1 has been degraded, resulting in an inhibition of APC/C^{Cdc20} that is independent of the SAC inhibitors and Emi1 (Song et al., 2004). However, a later report suggests that Cdc20 does not associate with RASSF1A, and thus the role of RASSF1A is still not clear (Liu et al., 2007). While the role of RASSF1A in mitosis remains unsolved, RASSF1A can regulate the G1/S transition by restricting Emi1 ubiquitylation by SCF ^{β -TrCP}. This restriction leads to Emi1 mediated APC/C inhibition (Whitehurst et al., 2008).

Nucleocytoplasmic transport factors Rae1 and Nup98 can form a complex with Cdh1 and specifically inhibit the ability of APC/C^{Cdh1} to ubiquitylate securin (Jeganathan et al., 2005). Recently, CUE domain-containing protein 2 (CUEDC2) was reported to inhibit Cdh1 by binding to Cdh1 through its KEN box; this inhibition leads to accumulation of Cyclin A and G1/S progression (Zhang et al., 2013). Intriguingly, CUEDC2 protein was reported to activate Cdc20 by binding with Cdc20 and releasing Cdc20 from MCC complex (Gao et al., 2011).

1.4 Identification of TRIM39

1.4.1 TRIM39 and Xnf7

Several years ago, our laboratory reported identification of a novel inhibitor of the APC/C in extracts from eggs of *Xenopus laevis* called Xnf7 (Casaletto et al., 2005). Xnf7 is an ~80 kDa protein, which has the ability to bind DNA and is suggested to function as a transcription factor, though no Xnf7-target genes have been reported (Li et al., 1994; Reddy et al., 1991) (Miller et al., 1991). Xnf7 contains a cytoplasmic retention domain (PRY-SPRY domain), a coiled-coil domain, a nuclear localization sequence, a N-terminal chromo domain and a RING domain (Beenders et al., 2007). It can be phosphorylated by Cyclin B/Cdk1 and MAPK. Phosphorylation of Xnf7 can change its subcellular localization, which in turn affects its ability to regulate the dorsal-ventral patterning of the *Xenopus* embryo. The association of Xnf7 with specific cellular

structures (the spindle or centrosomes) is also dependent on its phosphorylation state. Xnf7 associates with the mitotic spindle during mitosis and with the centrosomes during interphase (Li et al., 1994). It has also been reported to associate with the active transcription units of RNA polymerase II during oogenesis and the association requires the formation of Xnf7 homotrimerization through its coiled-coil domain (Beenders et al., 2007). (El-Hodiri et al., 1997a; El-Hodiri et al., 1997b; Li and Etkin, 1993; Miller et al., 1991; Reddy et al., 1991). Xnf7 depletion leads to hypersensitivity of spindles to microtubule-depolymerizing agents. The C-terminus of Xnf7 mediates binding with microtubules, which contributes to microtubule organization and spindle integrity (Maresca et al., 2005).

Xnf7 was previously identified as a Cyclin B interacting protein in our laboratory. We later found that recombinant Xnf7 can inhibit APC/C mediated ubiquitylation in vitro and decrease Cyclin B degradation in *Xenopus* egg extract. This inhibitory ability is dependent on its RING-dependent ligase activity. We also found that antibody depletion of Xnf7 can override SAC signaling, permitting Cyclin B degradation (Casaletto et al., 2005).

In searching for a human homolog of the *Xenopus* Xnf7 protein, we identified two proteins, TRIM39 and TRIM69, that share 42% and 46% sequence homology to Xnf7, respectively. In this thesis, I describe our in depth characterization of Trim 39 function.

1.4.2 TRIM39 function

TRIM39 was initially cloned from a human testis cDNA library in 2000. The cDNA produces a 518-amino acid protein and shares 98% identity with the mouse TRIM39 sequence. The gene is mapped to the class I region of the human MHC (major histocompatibility complex) on chromosome 6. Northern blotting showed that the gene is ubiquitously expressed, with strong expression in the testis, skeletal muscle and spleen (Orimo et al., 2000). Alternate splicing results in two transcript variants encoding different isoforms: 518 amino acids (isoform 1) or 488 amino acids (isoform 2). Isoform 2 lacks exon 7 when compared to isoform 1. Of note, we used isoform 2 for all overexpression and protein purification experiments in the dissertation whereas our shRNA can target both isoforms. Later in 2007, Robert *et al.* cloned a splice variant cDNA (TRIM39R) which contains the COOH-terminal portion of Rpp21, a component of RNase P (Roberts et al., 2007). The TRIM39R cDNA product was found to interact with cGMP-dependent protein kinase I (PKGI) by the yeast two-hybrid method. This group also reported that both TRIM39 and TRIM39R can interact with PKGI. However, the function of these interactions is still unclear (Fig. 1.7).

TRIM39 belongs to the Tripartite Motif (TRIM) family and contains a RING domain, two B-boxes, a coiled-coil domain and a PRY-SPRY domain. The RING motif is a linear sequence of conserved cysteine and histidine residues that can bind two zinc

ions in a manner similar to a zinc finger motif. The B-box also possesses a zinc ion binding ability, and the coiled-coil domain can mediate homomeric or heteromeric interactions with other proteins. Most TRIM proteins play a role in post-translational modification; such modifications include: ubiquitylation, sumoylation (small ubiquitin modifier), and ISGylation (interferon (IFN)-induced 15-kDa protein encoded by IFN-stimulated gene 15). They have been reported to be involved in tumor formation, apoptosis, immune responses, and transcriptional regulation (Ikeda and Inoue, 2012). For example, TRIM19, TRIM24, TRIM25, and TRIM27 have been linked to tumor initiation and progression. Mutation of several TRIM family genes has been found to lead to disease formation. For example, mutations in TRIM18 are associated with X-linked Optiz G/BBB syndrome; mutations in TRIM20 are linked to familial Mediterranean fever; mutations in TRIM37 are related to mulibrey nanism and mutations in TRIM32 are connected to limb girdle muscular dystrophy type 2H (Ryu et al., 2011)

There are few studies regarding the function of TRIM39. TRIM39 has been suggested to be associated with Behçet's disease as the presence of a single nucleotide polymorphism (SNP) located on exon 9 of TRIM39 occurs with disease development (Kurata et al., 2010). Another study suggests a role for TRIM39 in apoptosis. Using the yeast two-hybrid method, TRIM39 was found to interact with modulator of apoptosis 1

(MOAP-1). MOAP-1 protein was previously identified as a Bcl-2-associated protein X (Bax) interactor, and MOAP-1 can facilitate Bax activation to promote apoptosis (Tan et al., 2005). When TRIM39 is overexpressed, its interaction with MOAP-1 stabilizes MOAP-1 levels and enhances etoposide-induced Bax-mediated apoptosis. Accordingly, TRIM39 knockdown dampens cell death following etoposide treatment. These findings suggest that TRIM39 enhances MOAP-1 protein stabilization, possibly by inhibiting the regulator(s) of MOAP-1 (Lee et al., 2009).

1.5 Apoptosis

Apoptosis is an elaborate mechanism that serves to maintain cell homeostasis. It is important for development and multi-cellular function since it can eliminate cells that are potentially detrimental to the organism or cells that are inactive in a specific development stage. Misregulation of apoptosis is involved in both cancer progression and neurodegeneration. Apoptosis is characterized by fragmented chromatin, reduced cell volume and cell membrane blebbing, which exposes phosphatidylserine. This phospholipid will attract macrophages to execute phagocytosis of the cells, thereby preventing the release of cellular contents which might otherwise damage surrounding cells. (Afford and Randhawa, 2000; Kerr et al., 1972) (Elmore, 2007; Martin et al., 1995).

Apoptosis is executed by a subset of cysteine dependent aspartate-directed proteases known as caspases. In healthy cells, caspases are translated as inactive

zymogens which are activated via proteolytic cleavage and/or oligomerization upon receiving a death stimulus. There are two classes of caspases: initiator caspases (caspase-8, 10, 9, 2) and effector caspases (caspase-3, 7, 6.) Initiator caspases automatically activate when they interact with adaptor proteins after receiving pro-death signals. These activated initiator caspases go on to relay these signals to effector caspases, which consequently become activated through proteolytic cleavage. These activated effector caspases will go on to cleave their substrates, thus promoting apoptosis (Fuentes-Prior and Salvesen, 2004; Kumar, 2007; Nicholson and Thornberry, 1997; Nunez et al., 1998; Stennicke and Salvesen, 1998).

1.5.1 Apoptosis pathways

Initiator caspases can be activated by either an extrinsic or intrinsic pathway (Fig. 1.8). The extrinsic pathway is initiated when an extracellular ligand such as Fas, tumor necrosis factor α (TNF- α), or TNF related apoptosis inducing ligand (TRAIL) binds to its specific receptor, leading to activation of caspase-8 or caspase-10. After the ligands bind the Cys rich extracellular subdomains of these receptors, the receptors will subsequently trimerize and activate. Subsequent signaling is mediated by the death domain (DD) containing cytoplasmic part of the death receptor. This DD domain interacts with a DD domain on an adapter molecule like Fas-associated death domain protein (FADD) or Tumor necrosis factor receptor type 1-associated DEATH domain (TRADD) to form the

death inducing signaling complex (DISC). The death effector domain (DED) of FADD will then recruit procaspase-8 or procaspase-10 to the DISC to facilitate activation of caspase-8 or caspase-10. The initiator caspases can then go on to directly cleave and activate caspase-3, which in turn mediate the proteolytic cleavage of various substrates and ultimately cell death. Caspase-8 can also cleave Bid (t-Bid) and engage the intrinsic pathway to induce apoptosis efficiently (Ashkenazi and Dixit, 1998; Danial and Korsmeyer, 2004; Kischkel et al., 2000; Kischkel et al., 2001) (Fulda and Debatin, 2006) (Wajant, 2002) (Wang and El-Deiry, 2003) (Slee et al., 1999).

The intrinsic pathway senses intracellular stresses, such as DNA damage, oxidative stress, and nutrient deprivation, and is mediated by the B cell lymphoma 2 (Bcl-2) protein family. These proteins control mitochondrial outer membrane permeabilization (MOMP) and cytochrome *c* release (Waterhouse and Green, 1999). Cytochrome *c* then binds Apaf-1 to assemble the apoptosome wherein caspase-9 becomes activated (Li et al., 1997; Srinivasula et al., 1998). Caspase-9 then activates the downstream effector caspases, caspase-3, -7, and -6. Besides caspase-9, caspase-2 is also considered to be an initiator caspase in response to certain apoptotic stimuli, including heat shock, DNA damage, nutrient deprivation and ER stress (Krumschnabel et al., 2009). It has been reported that the "PIDDosome", which contains the p53-Induced protein with a Death Domain (PIDD) and an adaptor protein, the RIP-associated

ICH-1/CED3 homologous protein with a Death Domain (RAIDD), mediates caspase-2 dimerization and activation in response to DNA damage. Once activated, caspase-2 will cleave Bid (t-Bid) and t-Bid will then promote cytochrome *c* release (Janssens and Tinel, 2012).

1.5.2 Bcl-2 family and apoptosis

As mentioned previously, Bcl-2 family proteins are critical regulators of cytochrome *c* release and therefore control the balance between cellular life and death. This balance is influenced by the interaction between pro-apoptotic (e.g., Bax, Bak, or the Bcl-2 homology domain 3 (BH3)-only proteins Bid, Bim, Bad, or PUMA) and anti-apoptotic (Mcl-1, Bcl-2, or Bcl-xl) Bcl-2 family proteins (Kelekar and Thompson, 1998) (Adams and Cory, 2007) (Fig. 1.9). The anti-apoptotic proteins promote cell survival by binding through BH domains and blocking the function of pro-apoptotic proteins. BH3-only proteins promote apoptosis by either activating Bax/Bak or neutralizing anti-apoptotic proteins from sequestering Bax/Bak (Westphal et al., 2011) (Kelekar et al., 1997; Korsmeyer, 1999; Wang et al., 1996).

Bax and Bak are direct regulators of MOMP; they can translocate and insert into the mitochondrial outer membrane, oligomerize, and form pores. Bak is constitutively located at mitochondria whereas Bax is shuttled between the cytosol and mitochondria as a monomer. Upon apoptotic stimulation, Bid, Bim, and PUMA induce a

conformational change in Bax, allowing it to target the mitochondria and form oligomers, thereby enabling cytochrome *c* release (Kim et al., 2009) (Westphal et al., 2011) (Walensky and Gavathiotis, 2011).

MOAP-1 protein is reported to bind Bax through an interaction between MOAP-1's BH3 like domain and all three BH domains of Bax upon apoptotic stimuli. When it is overexpressed, it can trigger apoptosis (Tan et al., 2001.) When it is knocked down, either the intrinsic or extrinsic apoptosis pathways are compromised. MOAP-1 facilitates Bax- and tBid-mediated release of cytochrome *c* from isolated mitochondria (Tan et al., 2005). Moreover, it has been reported to cooperate with RASSF1A to activate Bax in response to activated K-Ras, TNF-alpha, or TRAIL stimulation (Foley et al., 2008; Vos et al., 2006). Under healthy conditions MOAP-1 is a short-lived protein, but its stability is increased upon intrinsic or intrinsic apoptotic stimulation (Fu et al., 2007).

1.6 p53

The tumor suppressor p53 was discovered in 1979 as a 53 kD protein that interacts with the SV40 large T antigen (Linzer and Levine, 1979). p53 is a crucial transcription factor that governs many biological activities such as cell cycle arrest, senescence, apoptosis, DNA damage responses, and metabolism. The importance of p53 can be demonstrated by the fact that nearly 50% of cancers contain TP53 (which encodes p53) inactivating mutations (Wade et al., 2010). In other cancers, TP53 is not mutated but

instead is silenced by deregulation of the p53 signal transduction pathway (Zuckerman et al., 2009). Accordingly, p53^{-/-} mice develop tumors within 6 months after birth and p53^{+/-} mice are also prone to form tumors, although more slowly. (Fuster et al., 2007). (Donehower et al., 1992)

1.6.1 The function of p53

p53 is an inactive and unstable protein under normal physiological conditions; however, it can be stabilized and induce a stress response following specific stimuli, including direct DNA damage, exposure to mitotic spindle toxic chemicals, hypoxia, oncogenic signaling, ribonucleotide depletion, and exposure to nitric oxide (Pluquet and Hainaut, 2001). Typically, these processes are attributed to transcriptional activity of nuclear p53. For example, p53 up-regulates cell cycle arrest-related genes such as p21 and 14-3-3 σ , or it promotes pro-apoptotic genes including Bax, Bak and PUMA. In addition, p53 can enhance autophagy by induction of DRAM and Sestrin2. p53 can also inhibit glucose metabolism via up-regulation of TP53-induced glycolysis and apoptosis regulator (TIGAR) expression. (Vogelstein et al., 2000) (Yu et al., 2001) (Reinhardt and Schumacher, 2012) (Giono and Manfredi, 2006; Levine and Abrams, 2008)

However, there is increasing evidence suggesting that cytosolic p53 also plays a non-genomic role in apoptosis. Cytosolic p53 can translocate to the mitochondrial outer

membrane and promote Bax/Bak activation through interaction with Bcl-2 and Bcl-xl. p53 has also been reported to interfere with the interaction between Bak and Mcl-2 by directly associating with Bak. There are also reports suggesting that mitochondrial p53 interacts with Bak and Bad to induce apoptosis. Moreover, cytosolic p53 has been shown to inhibit autophagy, probably through mechanisms related to LC3 or HMGB1 proteins. (Marchenko et al., 2000) (Mihara et al., 2003) (Chipuk et al., 2004) (Jiang et al., 2006) (Scherz-Shouval et al., 2010; Tasdemir et al., 2008) (Livesey et al., 2012) Figure 1.10 summarizes the role of p53 in cell cycle arrest and apoptosis.

1.6.2 Post-translational regulation of p53

Besides localization, p53 function and activity are regulated by post-translational modification. Post-translational modifications on different residues coordinately affect the interactors, stability, and target genes of p53, and many of these sites are conserved. Potential modifications of these sites include phosphorylation, acetylation, ubiquitylation, sumoylation, neddylation and methylation. These modifications of p53 vary in different cell lines and under various conditions, providing complex and intricate layers of p53 regulation.

Human p53 consists of five functional domains (Fig. 1.11). The N-terminal transactivation domain (TAD) is required for activation of transcription. Close to the transactivation domain is the proline-rich domain (PRD), which mediates protein-

protein interactions and regulates p53 stability. The DNA binding domain is central in the p53 molecule and binds to specific promoters of target genes; most mutations of p53 are found in this domain (Bode and Dong, 2004). The C-terminus of p53 consists of the tetramerization and the C-terminal regulatory domains. In cells without stress, p53 is inactive and maintained at low levels because MDM2 prevents transcription factors from binding to TAD and promotes p53 ubiquitylation and degradation (Toledo and Wahl, 2006 {Walker, 1996 #27} (Zhu et al., 1998) (Chang et al., 1995). Stress signals induce phosphorylation of p53 TAD and PRD domains (Ser-15, 20 and Thr 18). These modifications reduce MDM2 binding, thus stabilizing p53 and promoting p53 tetramerization. Tetramerization of 53 leads to higher nuclear retention and full transcriptional activity (Stommel et al., 1999) (Honda et al., 1997). Phosphorylation of these sites could also promote recruitment of more transcriptional co-activators.

As mentioned above, most phosphorylation modifications on p53 enhance its transcriptional activity; interestingly, some modifiable sites (ser-215, 376 and Thr-55) are constitutively phosphorylated and inhibit either transcriptional activity or DNA binding of p53 in unstressed cells. Moreover, other phosphorylation events can determine the function of p53. For instance, phosphorylation of Ser-46 mediates p53-dependent pro-apoptotic gene induction, but does not enhance expression of genes related to cell cycle

arrest (Stavridi et al., 2001) (Dai and Gu, 2010; Liu et al., 2004; Olsson et al., 2007; Taira et al., 2007).

Acetyl modifications can also affect p53 specificity for targeted genes in addition to stabilization of p53 by competing with ubiquitin for Lys sites. Lys-64 acetylation of p53 promotes the expression of genes involved in cell cycle arrest, such p21 (Knights et al., 2006). Lys-120 acetylation has been suggested to mediate p53-activated pro-apoptotic genes instead of cell cycle related genes. Additionally, this acetylation is required for p53-mediated dissociation of Bak and Mcl-2 to promote apoptosis (Sykes et al., 2006; Sykes et al., 2009).

1.6.3 Ubiquitin ligase of p53

The amount of p53 protein within a cell is strictly controlled by the ubiquitin-proteasome system to ensure proper cell proliferation and viability. As described previously, the RING-domain containing protein MDM2 is the major E3 ligase for p53. MDM2 mediates poly-ubiquitylation of p53 when the MDM2 level is high and mediates mono-ubiquitylation of p53 as a nuclear export signal when MDM2 is low (Li et al., 2003). Embryonic lethality in MDM2 null mice can be rescued by p53 knockout, indicating the importance of MDM2 in regulating p53 (Brooks and Gu, 2006; Jones et al., 1995; Montes de Oca Luna et al., 1995). Interestingly, MDM2 is also a transcriptional target of p53, thereby forming a negative feedback loop to regulate p53 activity. This

regulatory complexity is extensive as MDM2 can also autoubiquitylate, and the decision whether to target p53 or itself is influenced by ARF, which sequesters MDM2 away from p53 (Buschmann et al., 2000; Kubbutat et al., 1997; Pomerantz et al., 1998; Zhang et al., 1998). Moreover, CBP/p300 mediated-acetylation of MDM2 has been reported to reduce interaction between MDM2 and p53, and thus promotes p53 accumulation (Wang et al., 2004).

There are several other ubiquitin E3 ligases that can target p53. PIRH2, COP, CHIP, CARP1, CARP2, ARF-BP1, TRIM24, Synoviolin and Topors can polyubiquitylate p53, targeting it for degradation (Love and Grossman, 2012). In contrast, male-specific lethal-2 (Msl2) polyubiquitylates p53, but this ubiquitylation promotes its nuclear export instead of its degradation. WW domain-containing protein 1 (WWP1) E3 ligase can also ubiquitylate p53 and this modification changes the localization and stability of p53 by preventing MDM2-mediated p53 ubiquitylation. Similarly, Ubc13, an E2 conjugating enzyme, induces Lys63 polyubiquitylation on p53 to disrupt the interaction between p53 and MDM2. Moreover, this Lys63 ubiquitylation also signals nuclear export, decreases transcriptional activity, and reduces tetramerization of p53 (Brooks and Gu, 2011; Love and Grossman, 2012). Surprisingly, the transcription factor E4F1 behaves as an atypical E3 ligase (without RING or HECT domain) and polyubiquitylates p53, increasing the chromatin association of p53. This association regulates p53-dependent cell cycle arrest,

further demonstrating that post-translation modifications modulate the function of p53 (Le Cam et al., 2006). In this thesis, I describe the p53-ubiquitylating activity of TRIM39. Table 2 summarizes all the reported E3 ligases of p53.

Table 1. APC/C Inhibitors

Inhibitor	Species	Proposed to inhibit	Proposed mechanism
Cdk1	Sc, Sp, Hs	APC/C ^{Cdh1} in S and G2	Cdh1 phosphorylation
Cdk1	Xl	APC/C ^{Cdc20} in prometaphase	Cdc20 phosphorylation and interaction with Mad2
APC/C^{Cdh1}	Hs, Sc	APC/C ^{Cdc20} in anaphase	Cdc20 degradation
SCF	Hs	APC/C ^{Cdh1} in S phase	Cdh1 degradation
Mad2	Sc, Hs	APC/C ^{Cdc20} in prometaphase	Inhibition of Cdc20 substrate release
Mad2B	Xl, Hs	APC/C ^{Cdh1}	Inhibition of Cdh1 substrate release
BubR1	Hs	APC/C ^{Cdc20} in prometaphase	Cdc20 sequestration
Bub1	Hs	APC/C ^{Cdc20} in prometaphase	Cdc20 phosphorylation
MAPK	Xl	APC/C ^{Cdc20} in prometaphase	Cdc20 phosphorylation
Emi1	Xl, Hs	APC/C ^{Cdh1} in S and G2 phases	Substrate binding competition, inhibition of ubiquitin chain elongation, inhibiting ubiquitin transfer and interrupting E2-E3 association.
Rca1	Dm	APC/C ^{Cdc20} in S and G2 phases	Unknown
Emi2	Xl, Hs	APC/C ^{Cdc20} in meiosis II	Inhibiting ubiquitin transfer from E2 to substrate
Acm1	Sc	APC/C ^{Cdh1} in S and G2	Unknown. Forms complex with yeast 14-3-3 homologs (Bmh1/2)
Mes1	Sp	APC/C ^{Cdc20} during meiosis exit	Substrate binding competition
Mnd2	Sc	APC/C ^{Ama1} in meiosis I	Unknown
Rassf1A	Hs	APC/C ^{Cdc20} in mitosis	Cdc20 binding
Xnf7	Xl	APC/C ^{Cdc20} in mitosis	Unknown, ligase activity required
RAE1-NUP98	Mm	APC/C ^{Cdc20} in prometaphase	Interaction with Cdh1
Apoptin	CAV	APC/C ^{Cdh1}	APC1 binding
Unknown	HCMV	APC/C ^{Cdh1} in G0 phase	Inhibition of APC/C and Cdh1 interaction
CUEDC2	Hs	APC/C ^{Cdh1}	Substrate binding competition
E4orf4	HAV	APC/C ^{Cdc20}	PP2A recruitment
TRIM39	Hs	APC/C ^{Cdc20} and APC/C ^{Cdh1} in vitro	Unknown, ligase activity required

Mad2B is also known as MAD2L1. CAV, chicken anemia virus. Dm, *Drosophila melanogaster*. HAV, human adenovirus. HCMV, human cytomegalovirus. Hs, *homo sapiens*. MAP, mitogen-activated protein. Mm, *Mus musculus*. Mnd2, meiotic nuclear division protein-2. RAE1, Rab escort protein-1. RCA1, regulator of Cyclin A-1. Sc, *Saccharomyces cerevisiae*. Sp, *Schizosaccharomyces pombe*. Xl, *Xenopus laevis*. Xnf7, *Xenopus* nuclear factor 7. (Adapted and modified from (Peters, 2006))

Table 2. E3 Ubiquitin Ligase of p53

E3 ligase	E3 domain	impact on p53
MDM2	RING	Degradation, export, transcription
Pirh2	RING	Degradation, tetramerization
ARF-BP1	HECT	Degradation
E4F1	??	Transcription
CHIP	U box	Degradation
TRIM24	RING	Degradation, export, transcription
Cul1/Skp2	RING	Degradation
Cul4a/DDB1/Roc	RING	Degradation
Cul5	RING	Degradation
Cul7	RING	Export, transcription
Msl2	RING	Export
WWP1	HECT	Export, transcription, protein accumulation
Synoviolin	RING	Degradation, export, transcription
TOPORS	RING	Degradation,transcription
Cop1	RING	Degradation,transcription
CARP1/2	RING	Degradation
Ubc13	??	Export, transcription, tetramerization
TRIM39	RING	Degradation

Adapted from (Love and Grossman, 2012)

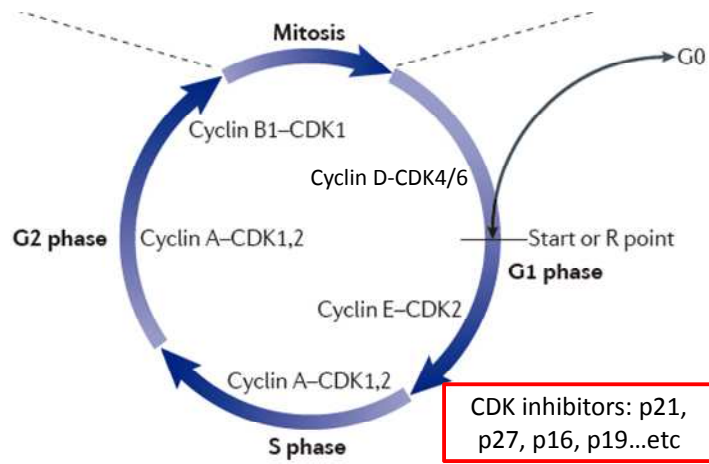


Figure 1. 1: The cell cycle.

Figure 1.1: Cell cycle is divided by G1, S, G2 and mitosis phases. Each phase is controlled by Cyclin/Cyclin-dependent kinase (Cdk) complex. Cdk activity can be modulated by various inhibitors such as p21, p27, p16 and p19. Figure is adapted and modified from (Pines, 2011).

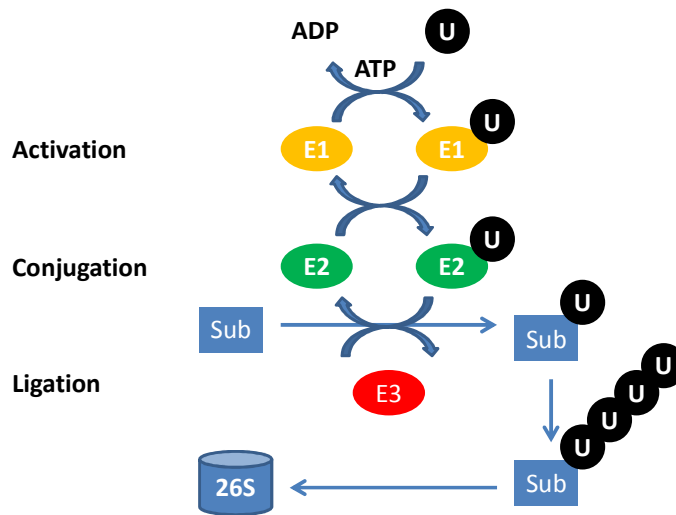


Figure 1. 2 Ubiquitin proteasome pathway.

Figure 1.2: Ubiquitylation is activated by E1 activating enzyme which utilizes ATP to form a thioester linkage between ubiquitin (U) and Cys on E1. E2 conjugating enzyme catalyzes the transfer of ubiquitin from E1 to Cys of E2. E3 ligase functions as substrate recognition molecule and interacts with both substrate and E2 to catalyze the transfer of ubiquitin. One cascade adds one ubiquitin molecule on the substrate and after several rounds, polyubiquitylation will mark the substrate to 26S proteasome for destruction. (modified from (Wasch and Engelbert, 2005))

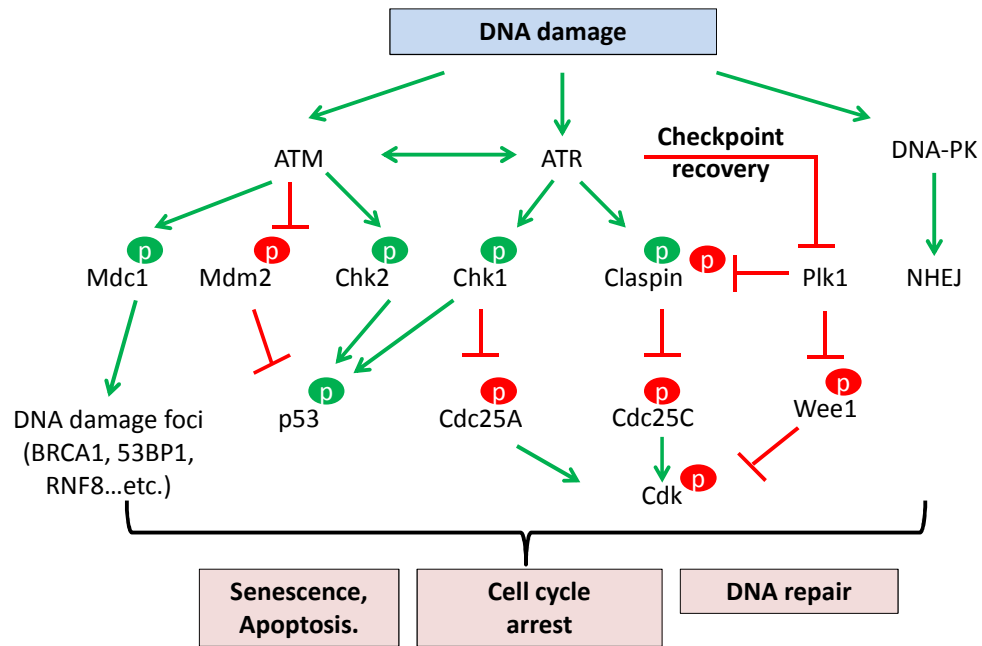


Figure 1. 3: DNA damage response.

Figure 1.3: A schematic diagram of DNA damage response. Green lines indicate activation. Red lines indicate inhibition by phosphorylation or by ubiquitin-proteasome system. Encircled p indicates phosphorylation. NHEJ: non-homologous end joining. (Modified from (Harper and Elledge, 2007))

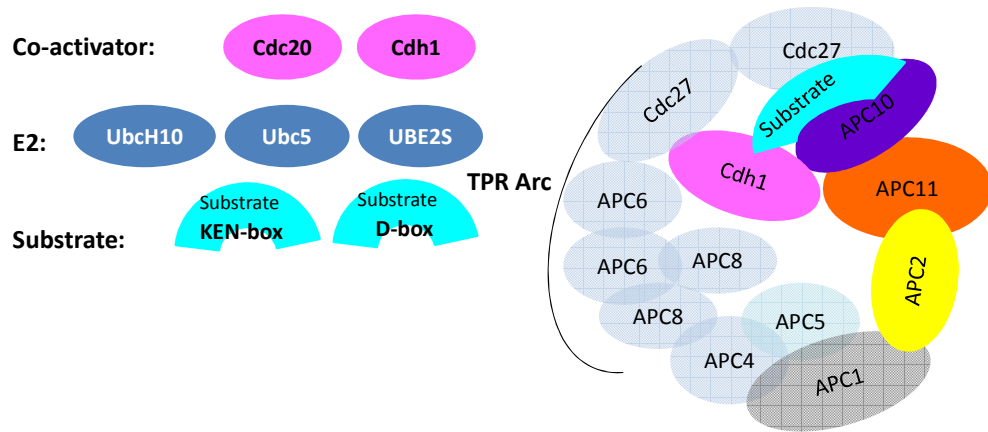


Figure 1. 4: APC/C components and structure.

Figure 1.4: Left: Different choices of co-activator, E2 conjugation enzyme and substrate motif of APC/C complex. Right: diagram cartoon represent the structure of APC/C^{Cdh1} complex resolved from cryo-EM map. The light gray molecular composes TPR domain containing subunit. Colorful molecular constitutes substrate docking site and enzyme activity site. This diagram is modified from (Schreiber et al., 2011.)

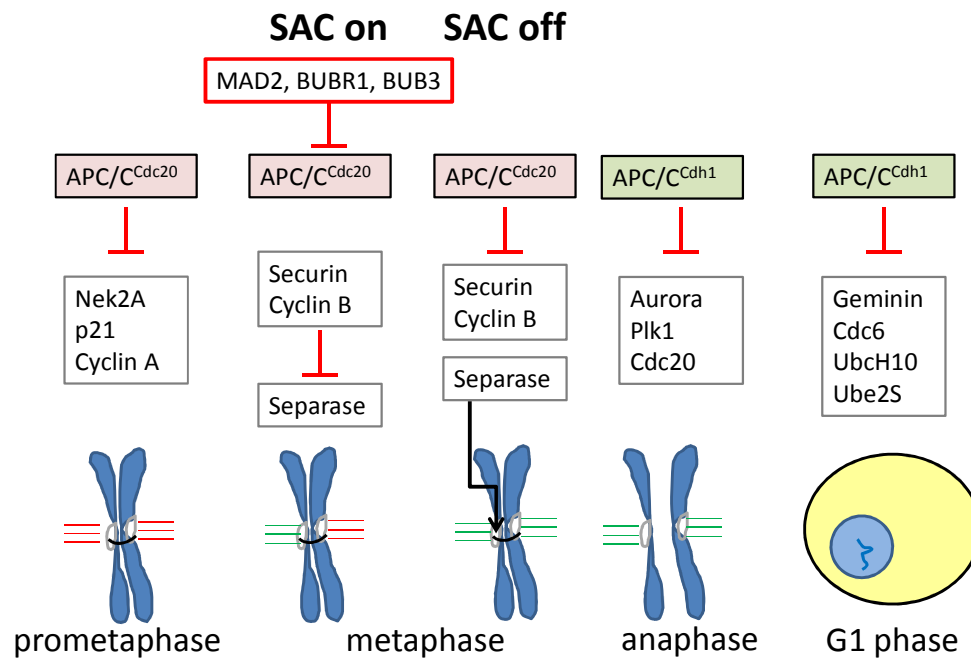


Figure 1. 5: APC/C regulates cell cycle.

Figure 1.5: The substrates of APC/C in different cell cycle stages. APC/C^{Cdc20} is active during mitosis where it controls the proper attachment of chromosomes on microtubules. APC/C^{Cdh1} is active from anaphase to G1 phase. For details, please read the text.

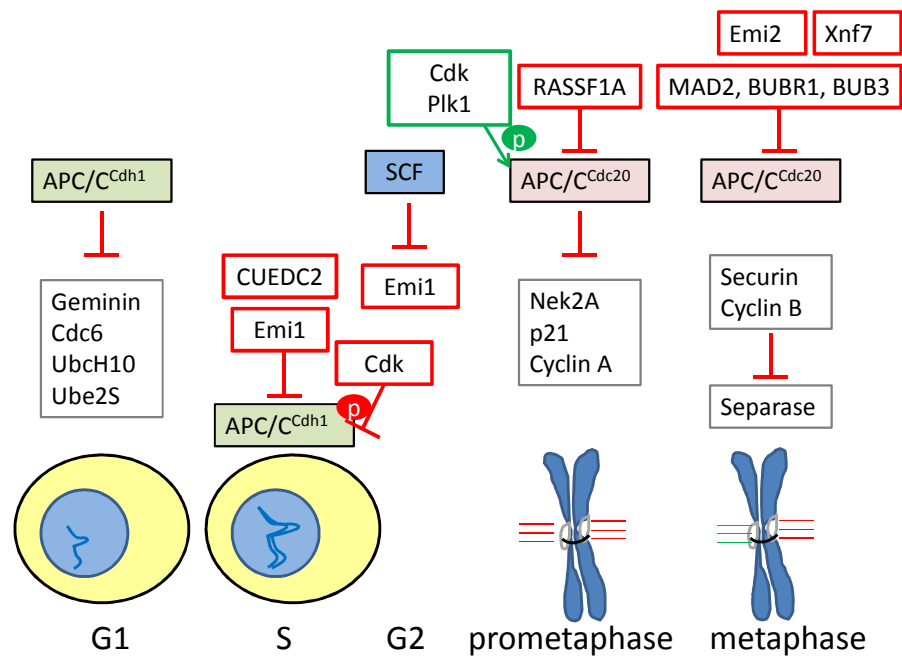


Figure 1. 6: Rugulation of APC/C activity.

Figure 1.6: APC/C activity is controlled by different means: phosphorylation, self-destruction and inhibitors. Each control is important for the timely activation of APC/C. Red box represents inhibition on APC/C; Green box represents activation on APC/C.

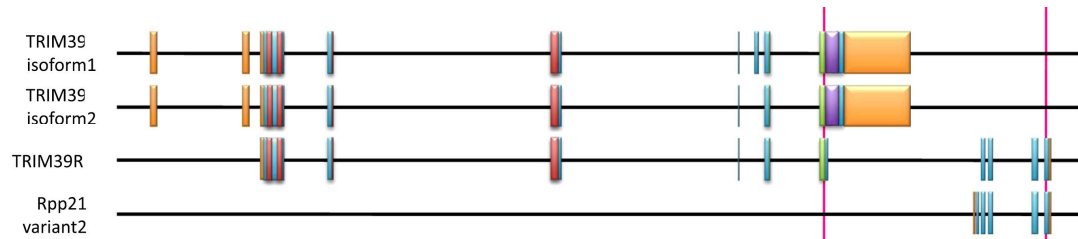


Figure 1. 7: The genome structure of TRIM39, TRIM39R, and Rpp21.

Figure 1.7: TRIM39 gene has three variants: two isoforms of TRIM39 full length and one fused with part of Rpp21 gene. One separate rectangle indicates an exon of transcripts. (Adapted from (Kurata et al., 2013))

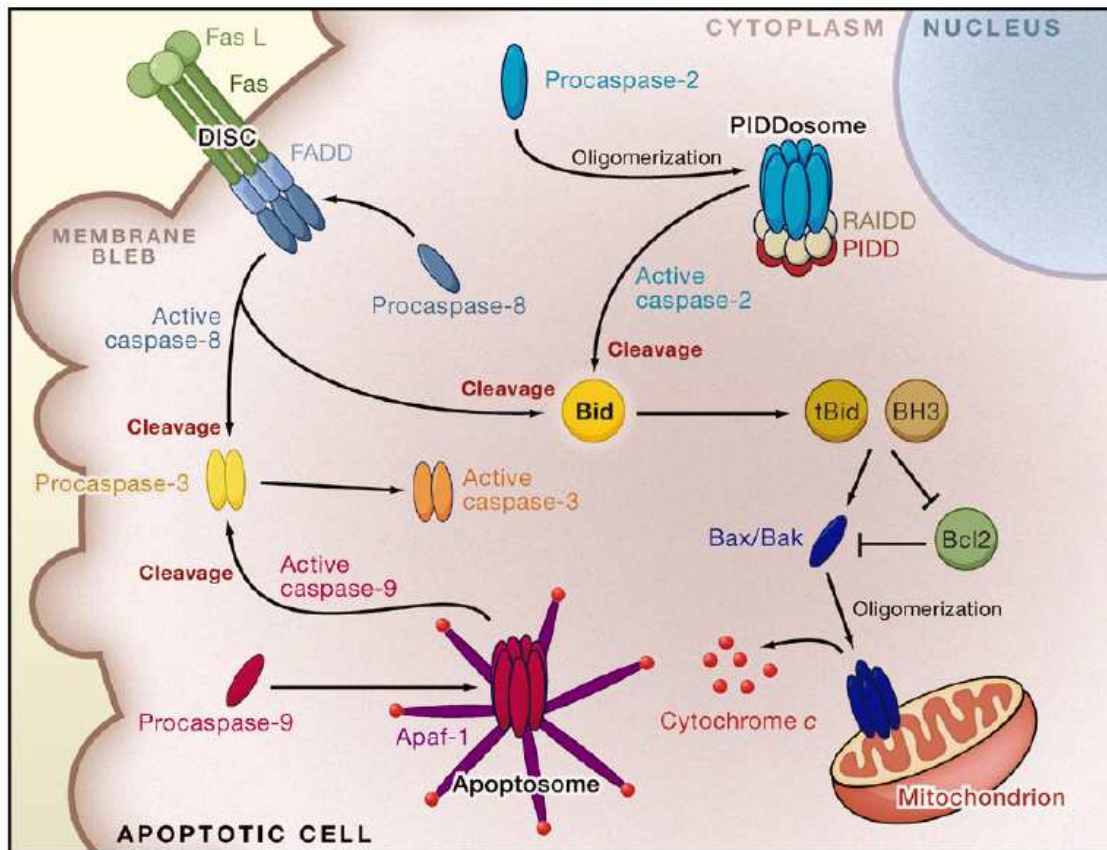


Figure 1. 8: Extrinsic and intrinsic apoptosis pathways.

Figure 1.8: Apoptosis can be initiated by extrinsic or intrinsic pathway. The extrinsic pathway is activated by death receptor to activate caspase-8. Intrinsic pathway includes PIDDosome activated caspase-2 or Bcl-2 family protein regulated cytochrome *c* release. Both pathways will activate caspase-9 through apoptosome and activate executor caspase. Adapted from (Kurokawa and Kornbluth, 2009)

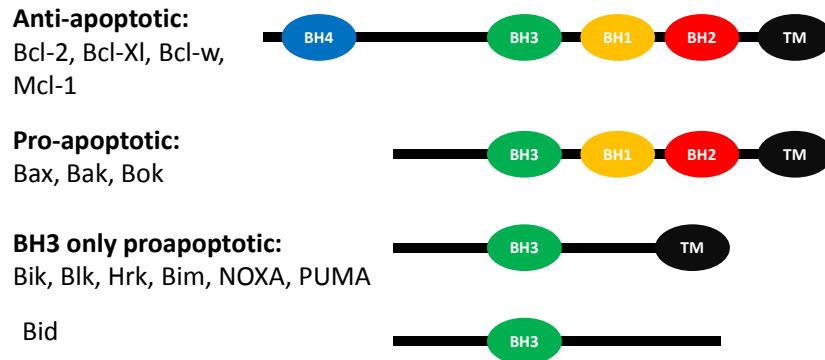


Figure 1. 9: Bcl-2 family proteins.

Figure 1.9: Bcl-2 family is divided by their roles in apoptotic promotion or anti-apoptotic function. They also can be divided by the number of BH (Bcl-2 homology) domain. TM: transmembrane domain. Modified from (Zimmermann et al., 2001).

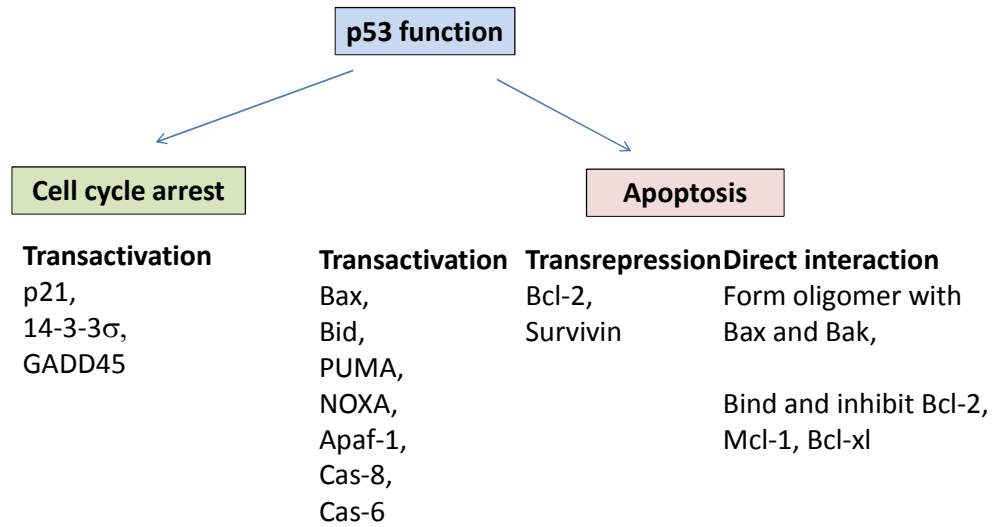


Figure 1. 10: p53 function in cell cycle and apoptosis.

Figure 1.10: p53 can modulate cell cycle and apoptosis through transcription activation or repression. p53 can also affect apoptosis by interacting with Bcl-2 family proteins. (Modified from (Zuckerman et al., 2009))

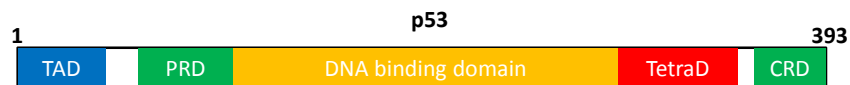


Figure 1. 11: Functional domain of 53.

Figure 1.11: p53 is a 393 amino acids length protein and has five functional domains. N-terminal transactivation domain (TAD) is required for transcription activation. Proline-rich domain (PRD) mediates protein-protein interactions and regulates p53 stability. DNA binding domains is involved in protein interaction and this region is regulated by C-terminal regulatory domain (CRD). Tetramerization domain (TetraD) mediates self-association of p53 and forms tetramer of p53 which can enhance transcription regulatory activity.

2. APC/C^{Cdh1}-mediated degradation of the Bax activator MOAP-1 controlled by the TRIM39 ubiquitin ligase

This chapter is adapted from (Huang et al., 2012)

2.1 Summary

Pro-apoptotic Bcl-2 family members, such as Bax, promote release of cytochrome *c* from mitochondria, leading to caspase activation and cell death. It was reported that MOAP-1, an enhancer of Bax activation induced by DNA damage, is stabilized by TRIM39, a protein of unknown function. In the chapter, we show that MOAP-1 is a novel substrate of the anaphase promoting complex (APC/C^{Cdh1}) ubiquitin ligase and that the influence of TRIM39 on MOAP-1 levels stems from the ability of TRIM39 (a RING domain E3 ligase) to directly inhibit APC/C^{Cdh1}-mediated protein ubiquitylation. Accordingly, siRNA-mediated knockdown of Cdh1 stabilized MOAP-1, thereby enhancing etoposide-induced Bax activation and apoptosis. These data identify TRIM39 as a novel APC/C regulator and provide an unexpected link between the APC/C and apoptotic regulation via MOAP-1.

2.2 Introduction

Apoptosis is critical for proper embryonic development, the response to cellular damage and overall organismal homeostasis. In the intrinsic apoptotic pathway, cell death stimuli promote release of mitochondrial cytochrome *c* to the cytoplasm, where it binds Apaf-1 to promote activation of the cell death protease.

Mitochondrial cytochrome *c* release is governed by Bcl-2 family proteins, including Bax and Bak, that trigger cytochrome *c* release through oligomerization at the mitochondrial outer membrane (Antonsson and Martinou, 2000; Eskes et al., 2000). Apoptotic signaling induces a conformational change in Bax and translocation to mitochondria where it can affect outer membrane permeabilization (Antonsson, 2001; Antonsson et al., 2001).

Modulator of Apoptosis-1 (MOAP-1, originally termed MAP-1) is a Bax-interacting protein whose knockdown inhibits apoptosis triggered by various stimuli (Tan et al., 2001). MOAP-1 association with Bax promotes Bax mitochondrial translocation and activation (Tan et al., 2005; Vos et al., 2006). Under non-apoptotic conditions, MOAP-1 undergoes constitutive degradation by the ubiquitin proteasome system, though the E3 ubiquitin ligase responsible for MOAP-1 ubiquitylation is unknown. Following receipt of certain cell death stimuli, MOAP-1 degradation is inhibited by TRIM39 (Orimo et al., 2000). Therefore, TRIM39 overexpression enhances etoposide-induced Bax-mediated apoptosis through stabilization of MOAP-1, while TRIM39 knockdown dampens cell death following etoposide treatment (Fu et al., 2007; Lee et al., 2009).

TRIM39 contains a RING domain, a B-box and a coiled-coil domain (Deshaies and Joazeiro, 2009). For a number of TRIM family members, the RING domain confers E3 ubiquitin ligase activity. However, since TRIM39 promotes MOAP-1 stabilization,

rather than degradation, it is clearly not directly responsible for MOAP-1 degradation. Rather, TRIM39 must in some way negatively regulate a MOAP-1-directed E3 ligase.

Analysis of TRIM39's primary sequence revealed it to be the closest mammalian homolog of a previously identified E3 ligase from *Xenopus laevis* known as Xnf7 (Casaletto et al., 2005). Xnf7 has several biological activities, including regulation of mitotic exit through its ability to inhibit the APC/C (Casaletto et al., 2005).

The APC/C is a multi-subunit E3 ubiquitin ligase that associates with one of two activators, Cdh1 or Cdc20. These activators mediate substrate recognition through consensus motifs present on substrates (e.g., the destruction box, RXXLXXXXN/D/E) (Pfleger and Kirschner, 2000). Cyclin A and Cyclin B1 ubiquitylation in M phase are mediated by the APC/C^{Cdc20}; in contrast, Cdh1 modulates the APC/C activity from M phase exit to G1, ubiquitylating Cdc20, Cyclin B1 and Geminin. The APC/C also controls degradation of a handful of other substrates involved in DNA replication, glycolysis and mitochondrial dynamics (Colombo et al., 2010; Sugimoto et al., 2008)

Here, we identify MOAP-1 as a novel APC/C^{Cdh1} substrate. MOAP-1 is degraded after anaphase by APC/C^{Cdh1} and this degradation is inhibited by TRIM39 acting on the APC/C. TRIM39 E3 ubiquitin ligase activity is required to block MOAP-1 (and other APC/C substrate) destruction. Furthermore, enhanced apoptosis following Cdh1 knockdown depends in part on MOAP-1. Thus, these data link the APC/C and apoptosis

and explain the previously reported connection between TRIM39 and the Bax activator, MOAP-1.

2.3 Material and method

2.3.1 Plasmids and protein preparation

HA-Cdh1 and Myc-MOAP-1 plasmids were kind gifts from M. Pagano (NYU) and V. C. Yu (IMCB, Singapore.) Recombinant maltose binding protein (MBP) fused to MOAP-1 was cloned from the pXJ40-Myc MOAP-1 plasmid into BamHI and SalI sites in the pMal vector and expressed in BL21 bacteria. MBP-TRIM39 was prepared from a construct purchased from ATCC. MOAP-1 mutants were prepared using QuikChange Site-Directed Mutagenesis Kits (Stratagene). In vitro translated ³⁵S-labeled MOAP-1, Cyclin B1, Geminin proteins were generated by using the TNT quick-coupled transcription/translation system (Promega).

2.3.2 Cell culture and synchronization

HeLa and HEK 293T cells were grown in DMEM with 10% fetal bovine serum at 37 °C. PC3 cells were grown in F12K medium. For nocodazole synchronization experiments, HeLa cells were double thymidine blocked using 2.5 mM thymidine and released into medium containing 100 ng/ml nocodazole after PBS washing for 16 h. To prepare cell extracts at different cell cycle stages, prometaphase cells were collected by shake-off, followed by washing with PBS and releasing into DMEM media for 0 h, 1 h or

4 h. The cells were lysed in hypotonic buffer (20 mM Hepes, pH7.7, 5 mM KCl, 1.5 mM MgCl₂, 1 mM DTT) on ice and released into the cell cycle at room temperature.

2.3.3 Transfection and siRNA

Fugene 6 (Roche) was used to transfect cells with plasmids according to the manufacturer's instructions. Lipofectamine RNAiMax (Invitrogen) was used to perform siRNA transfections. siRNA ON-TARGET plus SMARTpool targeted MOAP-1, TRIM39 were purchased from Dharmacon. Cdh1 siRNA (5'-GGAACACGCUGACAGGACA-3') was based on a previously reported effective sequence and purchased from Dharmacon (Song et al., 2011). siRNA directed against a non-mammalian protein, firefly luciferase (5'-CGUACGCGGAAUACUUCGA-3'), was used as the control siRNA.

2.3.4 Antibodies and immunoprecipitation

The following antibodies were used in this study: anti-CyclinB1, anti-HA, anti-Myc, anti-Actin and anti-Cdc27 (APC3) (Santa Cruz Biotechnology), anti-MBP (Abcam), anti-MOAP-1 (Sigma), anti-Bax (N20), anti-Bax (6a7) (BD Bioscience), anti-Cdh1 (Abcam), anti-caspase 3 (Cell Signaling).

Cells were lysed with EB buffer (20 mM Hepes (pH 7.4), 150 mM NaCl, 12.5 mM β -glycophosphate, 1.5 mM MgCl₂, 2 mM EGTA, 10 mM NaF, 2 mM DTT, 1 mM Na₃VO₄, 1 mM PMSF, 20 uM aprotinin and 0.5% triton X100) and extracts containing equal amounts of proteins were incubated with HA antibody (1ug) and protein A sepharose beads overnight at 4 °C. After washing three times in IP buffer (50 mM Tris (pH 7.5), 150

mM NaCl, 1 mM EGTA, 1mM EDTA, 1% Triton X-100 and 0.1% NP-40) and two times with IP buffer plus 150 mM NaCl and 0.02% triton X-100, samples were resolved by SDS-PAGE for immunoblotting.

2.3.5 TRIM39 in vitro ubiquitylation

0.25 μ M recombinant MBP-TRIM39 WT, C44A and C52A were separately incubated with 12.5 nM E1 and 250 nM E2 (UbcH5c or other E2s as indicated) and 10 μ M ATP at 30 $^{\circ}$ C for 1 h. For TRIM39 truncated mutant E3 ligase activity assay, 0.25 μ M recombinant MBP, MBP-TRIM39 WT, C44A and 1-104, and 1-336 were individually incubated with in vitro translated 35 S-labeled TRIM39 C44A, 12.5 nM E1 and 250 nM E2 (UbcH5c) and 10 μ M ATP at 30 $^{\circ}$ C for 1 h.

2.3.6 APC/C assay

The APC/C assay was performed as previously described (Tang et al., 2010). APC/C was precipitated using Cdc27 (APC3) antibody from M or G1 phase HeLa cells and then incubated with in vitro translated 35 S-labeled proteins, 240 μ M ubiquitin, 12.5 nM E1 and 250 nM E2 (UbcH5a, UbcH5c, UbcH10 or Ube2S as indicated), 10 μ M ATP and 2.25 μ M His-Cdh1 at 30 $^{\circ}$ C for 1 h. In some experiments, more than two E2s are added (each concentration is 250 nM) therefore the total E2 concentration is increased.

2.3.7 Pull down assay

2 μ g of MBP (0.25 μ M), MBP-MOAP-1 WT or D-box MT (0.1 μ M) were incubated with 0.75 μ g of His-Cdh1 in total volume 200 μ l IP buffer (50 mM Tris (pH 7.5), 150 mM

NaCl, 1 mM EGTA, 1mM EDTA, 1% Triton X100 and 0.1% NP-40) for 2 h at 4°C.

Amylose beads were added for another 1 h to pull down His-Cdh1, and washed five times with IP buffer. Samples were analyzed by immunoblotting.

2.3.8 Flow cytometry assay

Cells were trypsinized and washed with PBS. For sub-G1 analysis, cells were fixed with 70% ethanol in PBS in -20 °C overnight and stained with Propidium iodide (PI) for flow cytometry analysis.

2.4 Results

2.4.1 TRIM39 is an Xnf7-related E3 ubiquitin ligase

Previous work from our laboratory identified Xnf7 as a RING-domain containing E3 ligase and APC/C inhibitor in *Xenopus laevis* (Casaletto et al., 2005). In a blast search to uncover possible Xnf7 homologs in humans, we identified TRIM39, which is 36% identical and 55% similar to Xnf7. These proteins share a similar arrangement of N-terminal RING and B-box domains and contain similar PRY and SPRY domains within their C-terminal regions, suggesting that TRIM39 might share functional properties with Xnf7 (Fig. 2.1A). Using recombinant E1, E2 (UbcH5a, an E2 that could function with Xnf7), ubiquitin and TRIM39 as the only source of E3 activity, we found that TRIM39, like Xnf7, could autoubiquitylate. Several E2s could support TRIM39 autoubiquitylation (including UbcH5a), while others (most notably UbcH10, an E2 used by the APC/C)

could not (Fig. 2.2A). Moreover, RING-domain mutants expected to disrupt zinc binding (C44A and C52A) were deficient in enzymatic activity (Fig. 2.1B).

To determine whether TRIM39 could inhibit the APC/C, we added either Maltose-Binding Protein (MBP) or MBP-TRIM39 protein to lysates prepared from HeLa cells that had been synchronized in nocodazole, and then released (by wash-out of the nocodazole). As shown in Fig. 2.1C, Cyclin B1 was quickly degraded in the presence of recombinant MBP protein as these lysates exited from the mitotic arrest, while degradation of endogenous Cyclin B1 was nearly abolished by addition of recombinant MBP-TRIM39. This inhibition was not observed using the C44A mutant, suggesting that E3 ubiquitin ligase activity is required for APC/C inhibition (Fig. 2.1C). Indeed, Cyclin B1 was more rapidly degraded in the presence of the catalytically-inactive TRIM39 mutant, suggesting that this protein might interfere with the functioning of the endogenous protein. To confirm a direct role for TRIM39 in APC/C inhibition, we incubated APC/C immunoprecipitated from HeLa cells with MBP or MBP-TRIM39, E1, E2, ubiquitin and radiolabeled in vitro translated Cyclin B1. As shown in Fig. 2.1D, Cyclin B1 was well-ubiquitylated by the isolated APC/C and this activity was markedly reduced by incubation with MBP-TRIM39, as predicted for a direct APC/C regulator. To exclude the possibility that TRIM39 inhibited the APC/C by titrating the available E2, we supplemented the in vitro ubiquitylation reaction with UbcH10, which supports APC/C, but not TRIM39, E3 ligase activity. Under these conditions, TRIM39 still inhibited

ubiquitylation of Cyclin B1 by the APC/C (Fig. 2.1D). We also showed that APC/C inhibition does not result simply from titrating any other limiting factor (*e.g.* ubiquitin) away from the APC/C by isolating TRIM39 truncation mutants that retained auto-ubiquitylating activity (Fig. 2.2B), but lacked the ability to inhibit Cyclin B1 degradation (Fig. 2.2C). Furthermore, TRIM39 had no effect on an unrelated E3 ubiquitin ligase, DIAP1, which still auto-ubiquitylated in response to binding of its regulator, Reaper, in MBP-TRIM39-supplemented cell lysates released from nocodazole arrest (Fig. 2.2D). Thus, TRIM39 does not generally suppress ubiquitylation/degradation of proteasomal substrates.

2.4.2 TRIM39 inhibits MOAP-1 degradation during interphase

Since TRIM39 had been reported to modulate the ubiquitylation and degradation of the MOAP-1 and we had now found that TRIM39 could inhibit the APC/C, we hypothesized that MOAP-1 might be an APC/C substrate. To see if temporal degradation of MOAP-1 in the cell cycle was consistent with a role for the APC/C, we prepared HeLa cell extracts arrested at either prometaphase by nocodazole or further released for 0, 1 or 4 h, (representing prometaphase, metaphase or G1 extracts, respectively) and supplemented these extracts with radiolabeled, *in vitro* translated MOAP-1. MOAP-1 was not degraded in prometaphase or metaphase extracts, but was quickly degraded in G1 extracts, suggesting a role for Cdh1-activated APC/C in MOAP-1 degradation (Fig. 2.3A). Indeed, endogenous MOAP-1 levels were reduced in cells

released into G1 phase from nocodazole arrest, slightly delayed relative to endogenous Cyclin B1 degradation (Fig. 2.3B). This cell cycle-regulated degradation was similar to that seen for other APC/C substrates.

Since TRIM39 inhibited the APC/C (Fig. 2.1C and D), we asked whether this MOAP-1 degradation could be inhibited by TRIM39. Therefore, we added radiolabeled MOAP-1 or geminin (a control APC/C substrate) into cell lysates prepared from nocodazole-arrested cells and then pre-incubated with MBP-TRIM39 WT or MBP-TRIM39 C44A proteins during the release into G1 phase. Although MOAP-1 degradation was not as robust as geminin degradation, both proteins were degraded more rapidly in lysate containing MBP-TRIM39 C44A than in lysate containing MBP-TRIM39 (Fig. 2.3C). These data are consistent with the idea that APC/C promotes degradation of MOAP-1 and that this degradation can be inhibited by TRIM39.

2.4.3 MOAP-1 is an APC/C^{Cdh1} substrate

Since MOAP-1 was degraded in G1 cell lysates, we reasoned that the APC/C^{Cdh1} might ubiquitylate MOAP-1 to promote its proteasomal degradation. To assess this, we performed degradation assays using radiolabeled in vitro translated MOAP-1 as a substrate and found MOAP-1 degradation to be significantly slower in lysates in which Cdh1 had been knocked down using siRNA (Fig. 2.4A). Moreover, overexpression of HA-Cdh1 in conjunction with Myc-MOAP-1 in 293T cells led to a dose-dependent decrease in MOAP-1 levels, even in unsynchronized cells (Fig. 2.4B). While TRIM39

knock-down led to loss of MOAP-1 protein, MOAP-1 levels were restored by coordinate knock-down of both TRIM39 and Cdh1 (Fig. 2.5A). Furthermore, TRIM39 WT, but not C44A protein substantially inhibited ubiquitylation of MOAP-1 by the APC/C in vitro (Fig. 2.5B). Consistent with these findings, MOAP-1 and Cdh1 could co-immunoprecipitate (Fig 2.4C and D).

Our identification of TRIM39 as a candidate APC/C inhibitor was based on its sequence homology with Xnf7 (Casaletto et al., 2005). To determine if Xnf7 and TRIM39 were functionally similar, we knocked down TRIM39, resulting in nearly undetectable levels of MOAP-1; when WT, but not RING mutant Xnf7 was transfected into these cells, MOAP-1 was stabilized, suggesting that Xnf7 can, like TRIM39, inhibit APC/C-mediated MOAP-1 degradation (Fig. 2.5C).

In scanning the MOAP-1 sequence, we identified four potential D-box sequences that might confer recognition by Cdh1 (Fig. 2.6A). As shown in Fig. 2.6A, we mutated the underlined D-box consensus amino acids to Ala and transfected MOAP-1 wild type or D-box mutant constructs into 293T cells. MOAP-1 WT protein could be co-immunoprecipitated with HA-Cdh1 whereas MOAP-1 mutated at all four putative D-boxes (MOAP-1 MT) could not (Fig. 2.6B; we saw slight reduction of binding with each single D-box mutant, data not shown). Mutation of the D-box sequences caused a similar reduction in Cdh1/MOAP-1 interactions when recombinant His-Cdh1 was pulled down using MBP-MOAP-1 (WT or MT (Fig. 2.6C). Moreover, Myc-MOAP-1 MT

protein expressed in 293T cells was notably stabilized when compared with MOAP-1 WT after cycloheximide treatment (Fig. 2.6D). MOAP-1 WT was more robustly ubiquitylated than MOAP-1 MT both in cells overexpressing Cdh1 and treated with the proteasome inhibitor, MG132, and by the purified APC/C^{Cdh1} in vitro (Fig 2.6E and F). These data strongly suggest that MOAP-1 degradation results from APC/C^{Cdh1}-mediated D-box dependent ubiquitylation.

2.4.4 Depletion of Cdh1 enhances susceptibility to apoptosis in response to DNA damage

MOAP-1 has been reported to enhance apoptosis by activating Bax and knockdown of TRIM39 has been reported to decrease etoposide-dependent apoptosis. We predicted that loss of Cdh1 would lead to elevation of MOAP-1 (as this would disable the APC/C in much the same way as TRIM39 expression) and enhancement of etoposide-induced Bax activation. In agreement with this, in 293T cells treated with etoposide, the 6a7 antibody that recognizes conformationally active Bax, immunoprecipitated greater amounts of Bax in cells knocked down for Cdh1 than in cells treated with control siRNA. Cells treated with siRNA for MOAP-1 activated less Bax than control cells. Importantly, the increased Bax activation seen following knockdown of Cdh1 was significantly dampened following MOAP-1 knockdown (Fig. 2.7A; see Fig. 2.7B for protein levels following knockdown).

To evaluate the effect of Cdh1/MOAP-1 knockdown on chemotherapy-induced apoptosis, we treated PC3 prostate cancer cells with siRNA directed against Cdh1, MOAP-1 or both and monitored cell death by PI staining and flow cytometry. As shown in Fig. 2.7C and D, apoptosis was increased in Cdh1 knockdown cells in response to both etoposide and cisplatin treatment, but this increase was partially lost in cells that were ablated for both Cdh1 and MOAP-1 (see Fig. 2.7E for knockdown efficiency). This effect was even more marked with cisplatin than etoposide and the observed changes in cell death in response to cisplatin correlated well with caspase 3 cleavage (Fig. 2.7F). Thus, while there are likely additional APC/C^{Cdh1} substrates that contribute to the increased apoptotic response following loss of Cdh1, MOAP-1 degradation by APC/C contributes to setting the apoptotic threshold.

2.5 Discussion

2.5.1 TRIM39 as a novel inhibitor of APC/C

The data presented here identify TRIM39 as a new human E3 ligase able to regulate the APC/C and explain the previously reported link between TRIM39 and MOAP-1 abundance. Moreover, they demonstrate a role for the APC/C^{Cdh1} in controlling apoptosis through degradation of the Bax activator MOAP-1. Currently, it is not clear how TRIM39 inhibits the APC/C. Like *Xenopus* Xnf7, the E3 ligase activity of TRIM39 is required for this inhibition (Casaletto et al., 2005). TRIM39 can co-immunoprecipitate with the core APC/C (unpublished observations) and it is possible that it can

ubiquitylate an APC/C subunit or an associated regulator. Alternatively, TRIM39 may require autoubiquitylation to act as an APC/C inhibitor.

It would be of interest to determine whether TRIM39 functions at a particular cell cycle stage as other APC/C inhibitors have cell cycle-specific functions; for example, Emi1 inhibits APC/C during the G1 to S transition while Mad2 and BubR1 assume this role in prometaphase (Jackson, 2004; Skaar and Pagano, 2009). Our results indicate that TRIM39 can inhibit APC/C activity towards Cyclin B1, geminin, and MOAP-1, suggesting that TRIM39 can inhibit APC/C activity towards multiple substrates. Nonetheless, it remains possible that there is some substrate specificity to this inhibition.

2.5.2 APC/C links to apoptosis

Previous reports suggested that MOAP-1 was regulated at the level of protein stability. Our results indicate that the APC/C^{Cdh1} can ubiquitylate MOAP-1, providing a link between the APC/C^{Cdh1} and regulation of apoptosis. Indeed, MOAP-1 has been reported to be a short half-life protein (~25 mins) (Fu et al., 2007) and its stability is decreased once cells enter G1, the point where APC/C^{Cdh1} is known to act (Fig. 2.3). It is not yet clear if MOAP-1 has apoptotic effects that are cell cycle-specific or how cell cycle-specific degradation of MOAP-1 would help coordinate cell cycle status and apoptosis. Interestingly, etoposide treatment can stabilize MOAP-1 (Fu et al., 2007), suggesting that there are pathways linking the status of DNA or activation of DNA damage checkpoints to either TRIM39 or APC/C^{Cdh1}.

Recently, it was reported that APC/C^{Cdc20} ubiquitylates the anti-apoptotic Bcl-2 family protein Mcl-1 after a prolonged M phase arrest (Harley et al., 2010). In that MOAP-1 is stable during M phase and is not degraded until G1, it may participate in relaying the apoptotic signal after Mcl-1 is degraded in the event of prolonged M phase arrest. Under normal circumstances, at M phase exit, APC/C^{Cdc20} is degraded by APC/C^{Cdh1} followed by MOAP-1 degradation. At the same time, Mcl-1 can reaccumulate to prevent cell death. Although there are clearly multiple inputs into the cellular decision to live/die, this hand-off of apoptotic control between various short half-life proteins throughout the cell cycle may allow maximum flexibility/rapid cellular changes in response to cell cycle cues or cellular damage

Recently, it was reported that siRNA-mediated knock-down of Cdh1 in primary cortical neurons leads to apoptotic cell death (Almeida A et al., 2005). It has been hypothesized that the APC/C helps to maintain the differentiated state by preventing accumulation of Cyclin B1 and aberrant cell cycle re-entry. According to this scenario, death from loss of Cdh1 would follow a failure to suppress cell cycle progression (Aulia and Tang, 2006). These effects may more directly stem from stabilization of apoptotic regulators that are APC/C substrates. As MOAP-1 belongs to the human PNMA family and this family of proteins is particularly highly expressed in the brain, it would be interesting to determine if MOAP-1 accumulation also contributes to apoptosis in neurons ablated for Cdh1 (Schuller et al., 2005). Similarly, it has been

suggested that Cdh1 degradation after UV irradiation may promote cell death due to aberrant accumulation of Cyclin B1 (Liu et al., 2008). Our results raise the interesting possibility that Cdh1 degradation following irradiation might also lead to increases in MOAP-1 abundance to sensitize cells to UV-induced apoptosis.

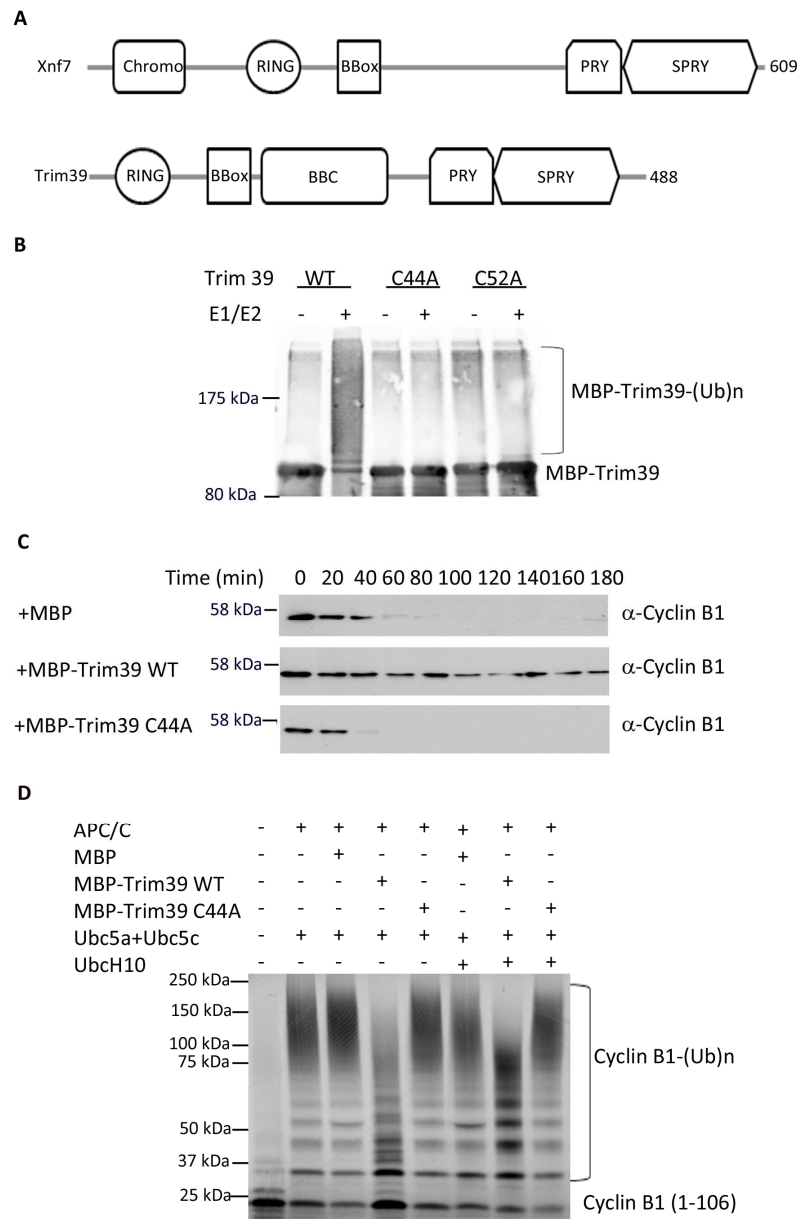


Figure 2. 1: TRIM39 is an Xnf7-related E3 ubiquitin ligase, and ligase activity is required for APC/C inhibition.

Figure 2.1: (A) Schematic representation of the domains found within Xnf7 and TRIM39 proteins. BBC represents the coiled-coil region C terminal to the B-Box domains. (B) In vitro ubiquitylation using recombinant TRIM39 WT, RING domain mutant, C44A, and C52A in the absence or presence of ubiquitin and E1/E2 (UbcH5a). (C) Cell lysates were prepared from HeLa cells synchronized after a 1-h release from nocodazole (allowing further release in vitro) and incubated with 1 μ M recombinant proteins, as indicated. Samples were collected and immunoblotted with Cyclin B1 antibody. (D) Radiolabeled in vitro translated Cyclin B1 fragment (1–106) was incubated with ubiquitin, E1 and E2 (UbcH5a, UbcH5c, or UbcH10, as indicated), ATP, His-Cdh1, and APC/C immunoprecipitated from HeLa cell G1 lysates using Cdc27 antibody. Reactions were supplemented with control buffer, MBP, MBP-TRIM39 WT, or C44A recombinant protein for 45 min. Samples were detected by SDS-PAGE and autoradiography.

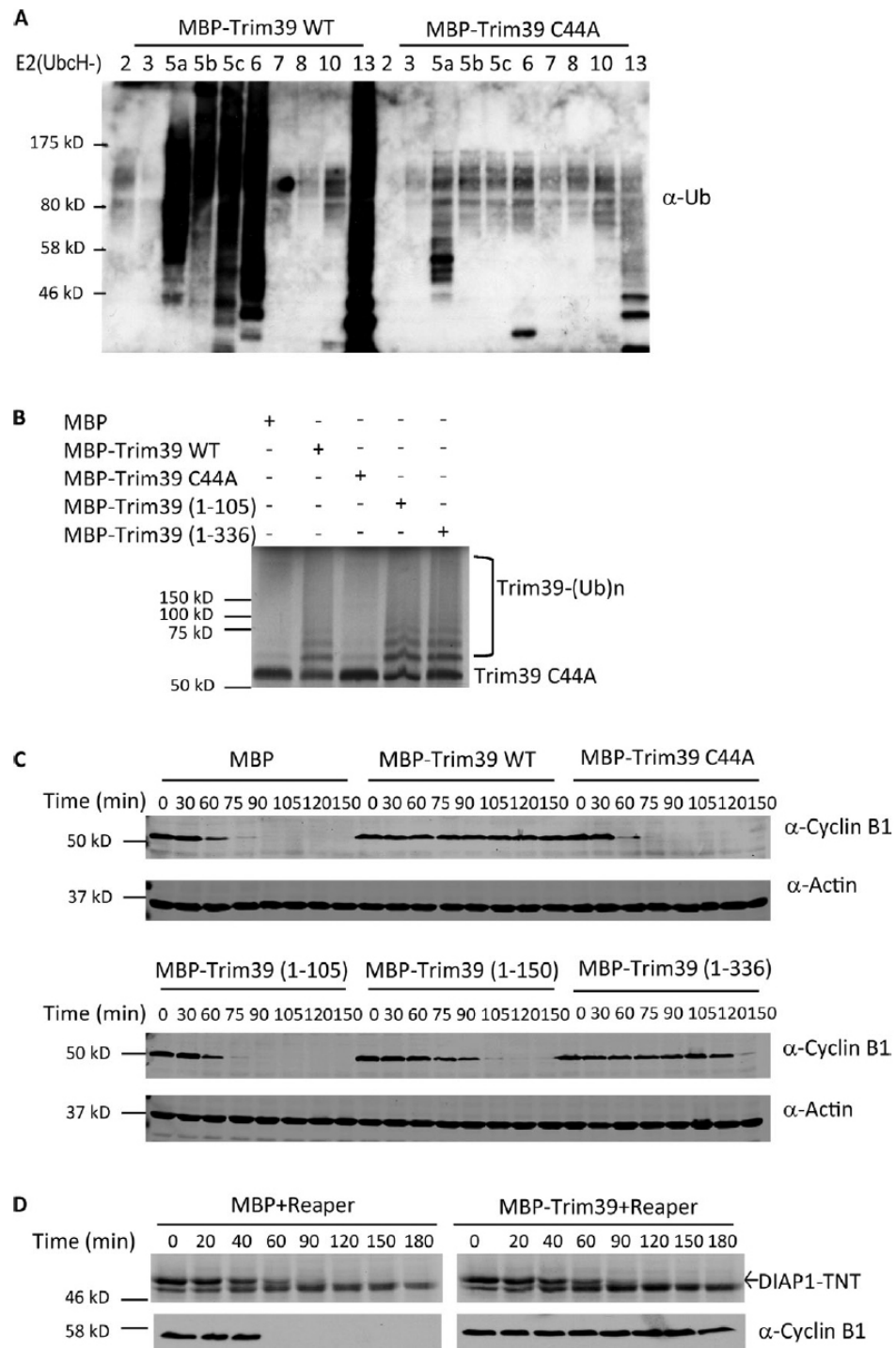


Figure 2. 2: Specificity of TRIM39

Figure 2.2: (A) TRIM39 can function with several E2s but cannot function with UbcH10. MBP-TRIM39 WT or C44A recombinant proteins were incubated with 240 μ M ubiquitin, 12.5 nM E1, 10 μ M ATP, and different E2 proteins in vitro for 1 h, an autoubiquitylation was detected by immunoblotting using anti-ubiquitin antibody. (B) 1 μ M MBP, MBP-TRIM39 WT, C44A, 1–105, and 1–336 fragments of TRIM39 were incubated with 240 μ M ubiquitin, 12.5 nM E1 and 250 nM E2 (UbcH5a and UbcH5c), 10 μ M ATP, and radiolabeled in vitro translated TRIM39 C44A protein for 30 min at 30°C, and samples were resolved by SDS-PAGE and autoradiography. (C) 1 μ M MBP, MBP-TRIM39 WT, C44A, 1–105, 1–150, and 1–336 recombinant proteins were incubated in nocodazole synchronized cell lysates, and samples were taken at the times indicated. Samples were resolved by SDS-PAGE and detected by anti-Cyclin B1 and anti-actin immunoblotting. (D) 1 μ M MBP and MBP-TRIM39 WT, 7.5 μ M Reaper recombinant proteins, and radiolabeled in vitro translated DIAP1 were incubated in nocodazole-synchronized cell lysates, and samples were taken at the times time indicated. Samples were resolved by SDS-PAGE gel and detected by autoradiography or anti-Cyclin B1 immunoblotting.

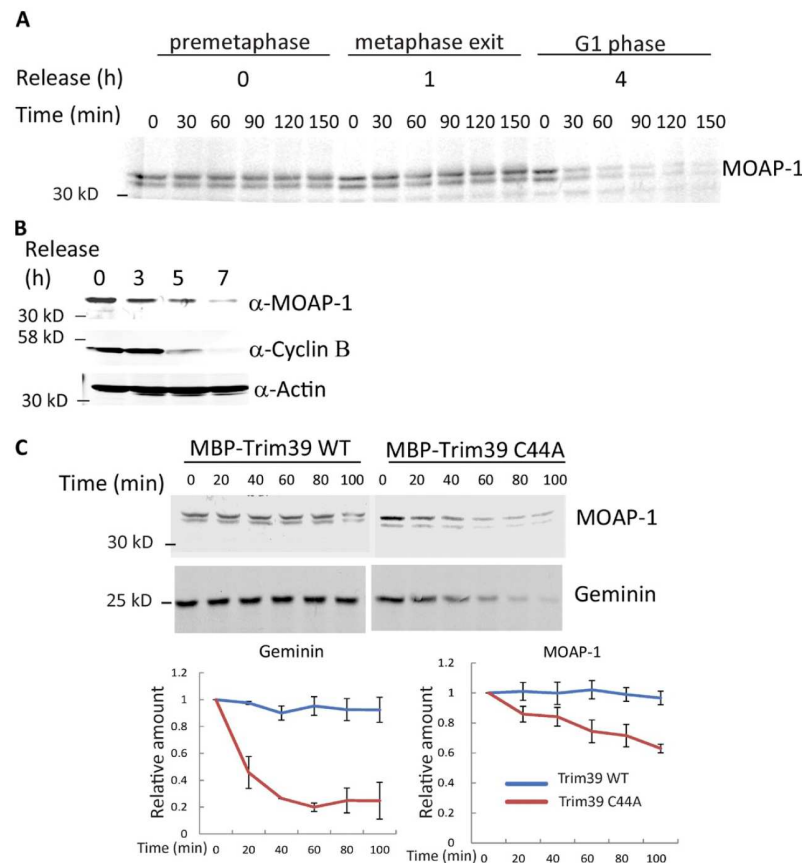


Figure 2.3: MOAP-1 protein is degraded in a cell cycle-specific manner, and TRIM39 attenuates the degradation.

Figure 2.3: (A) HeLa cells were synchronized at prometaphase with nocodazole and collected at the indicated times after release. Radiolabeled in vitro translated Cyclin B1 and MOAP-1 proteins were incubated in each lysate. Samples were taken at each time point, and reactions were stopped by addition of SDS loading buffer. (B) HeLa cells were synchronized with nocodazole and collected after release at the indicated times. Lysates were analyzed by immunoblotting with the indicated antibodies. (C) 1 μ M MBP-TRIM39 WT and C44A recombinant proteins were incubated in nocodazole-synchronized cell lysates for 3 h and then supplemented with in vitro translated, radiolabeled MOAP-1 or geminin. Samples were taken at each time point, and reactions were stopped by addition of SDS loading buffer. The line graphs below represent quantitation of three independent experiments. Error bars are SD.

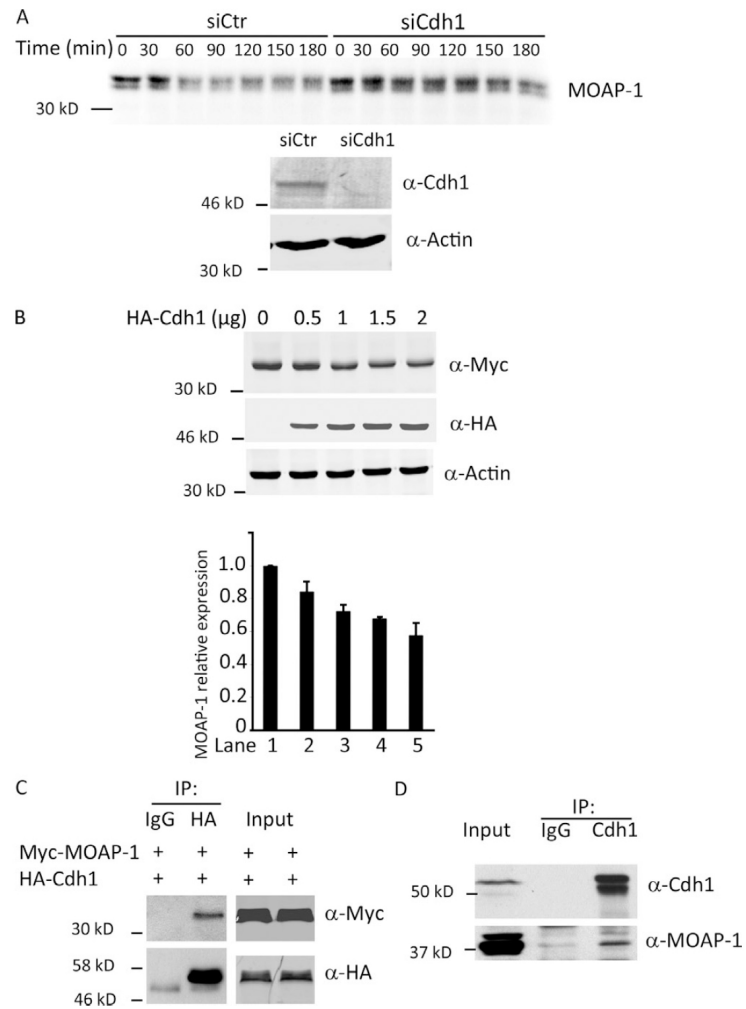


Figure 2. 4: Cdh1 regulates MOAP-1 stability.

Figure 2.4: (A, top) HeLa cells were treated with control or Cdh1 siRNA, arrested in nocodazole, released for 4 h, and lysed, and lysates were supplemented with radiolabeled in vitro translated MOAP-1 protein. Samples were withdrawn into SDS loading buffer at the indicated times and resolved by SDS-PAGE and autoradiography. (bottom) Immunoblot of Cdh1 from the Cdh1 siRNA-treated (siCdh1) cells above. siCtr, siRNA control. (B) 293T cells were transfected with 0.5 μ g Myc-MOAP-1 plasmid and increasing amounts of HA-Cdh1, as indicated. Cell lysates were collected and probed with anti-Myc, -HA, or -actin antibody. Relative Myc-MOAP-1 levels (normalized to levels seen in the absence of exogenous Cdh1 in lane 1) are quantitated below based on three repetitions of the experiment. Error bars are SD. (C) 293T cells were transfected with 0.5 μ g HA-Cdh1 and 1.5 μ g Myc-MOAP-1. Cell lysates were prepared and immunoprecipitated (IP) by normal IgG or anti-HA antibody and probed with anti-HA or -Myc antibody. (D) Anti-Cdh1 or control IgG immunoprecipitates from 20 μ M MG132-treated 293T cells were immunoblotted with anti-MOAP-1 or anti-Cdh1 antibody.

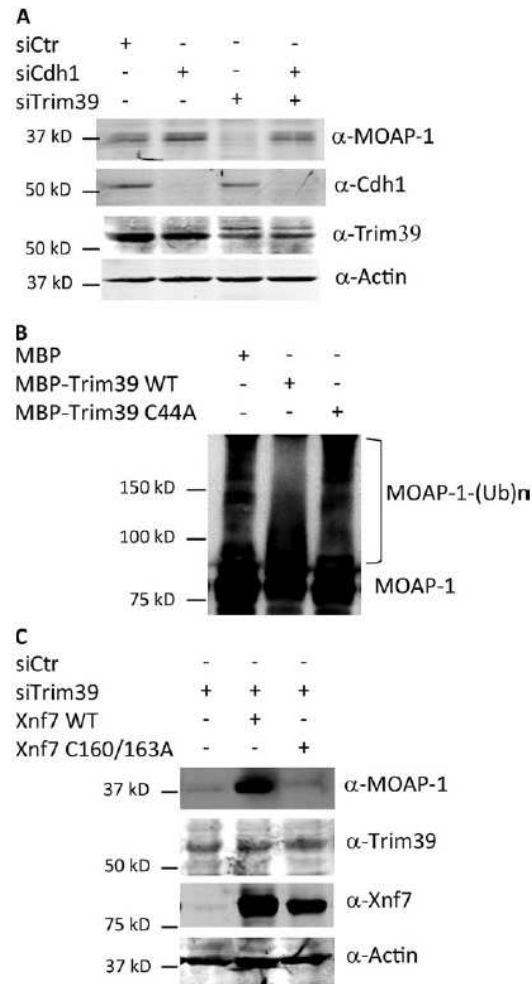


Figure 2. 5: Control of MOAP-1 ubiquitylation and degradation by TRIM39 and Xnf7

Figure 2.5: (A) 293T cells were transfected with control (siCtr), Cdh1 (siCdh1), or TRIM39 (siTRIM39)-targeted siRNA for 72 h. Cell lysates were immunoblotted with anti-MOAP-1, Cdh1, TRIM39, or actin antibodies. (B) 1 μ M recombinant MBP, MBP-TRIM39 WT, or C44A proteins were incubated separately with MBP-MOAP-1 protein, 240 μ M ubiquitin, 12.5 nM E1 and 250 nM E2 (UbcH5a, UbcH10, and Ube2S), 10 μ M ATP, and APC/C immunoprecipitated with anti-Cdc27 antibody from HeLa G1 cell lysates. Samples were immunoblotted with anti-MOAP-1 antibody. (C) Control or TRIM39 knockdown 293T cells were transfected with control, Xnf7 WT, or Xnf7 C160/163A for 48 h. Cell lysates were immunoblotted with anti-MOAP-1, TRIM39, Xnf7, or actin antibodies.

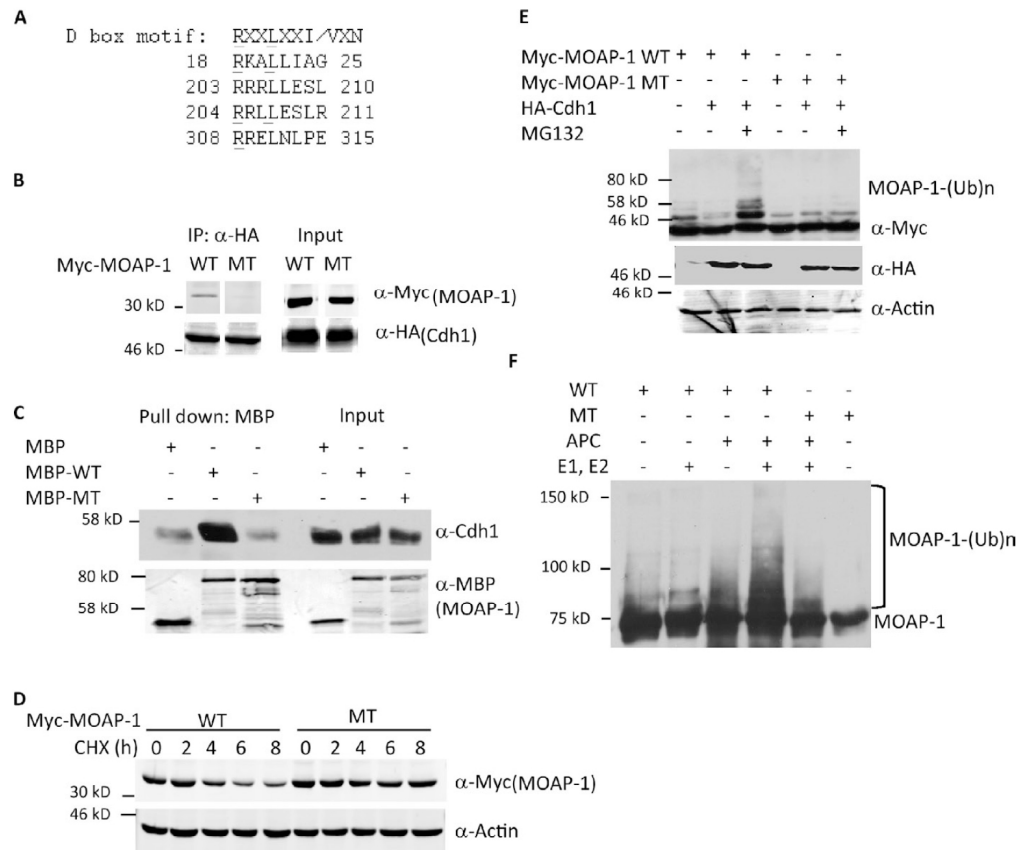


Figure 2. 6: MOAP-1 is an APC/CCdh1 substrate with D-box motifs.

Figure 2.6: (A) Sequences of putative D-boxes on MOAP-1. D-box consensus amino acids mutated to Ala and transfected MOAP-1 WT or D-box mutant constructs into 293T cells are underlined. (B) 293T cells were transfected with HA-Cdh1 and Myc-MOAP-1 WT or MOAP-1 MT. Cells were collected and immunoprecipitated (IP) by anti-HA antibody. Samples resolved by SDS-PAGE were immunoblotted using anti-Myc or -HA antibodies. (C) MBP or MBP-MOAP-1 WT or MBP-MOAP-1 MT was incubated with His-Cdh1 for 2 h at 4°C, and proteins were retrieved from the mixture using amylose beads. After SDS-PAGE, samples were immunoblotted with anti-MBP or -Cdh1 antibodies. (D) 293T cells were transfected with Myc-MOAP-1 WT or Myc-MOAP-1 MT and treated with 100 μ M cycloheximide (CHX). Cell lysates were prepared at the indicated times after cycloheximide treatment and immunoblotted with anti-Myc or -actin antibodies. (E) 293T cells were transfected with Myc-MOAP-1 WT or MT in the presence or absence of HA-Cdh1. After 48 h, cells were treated with 20 μ M MG132 for 16 h, and lysates were collected for immunoblotting with anti-Myc, -HA, or -actin antibodies. (F) Recombinant MBP-MOAP-1 WT or MT proteins were incubated with ubiquitin, E1 and E2 (UbcH10 and Ube2S), ATP, and APC/C precipitated from HeLa G1 cell lysates using anti-Cdc27 antibody. Samples were immunoblotted with anti-MOAP-1 antibody.

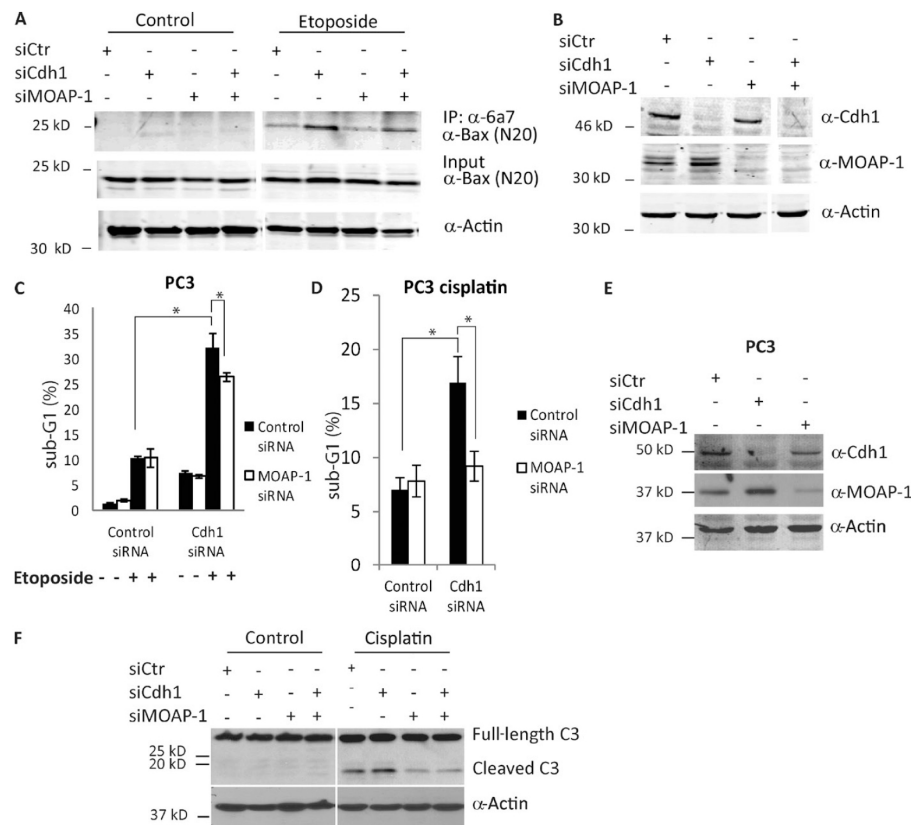


Figure 2. 7: Higher Bax activation in Cdh1 knockdown cells as a result of MOAP-1.

Figure 2.7: (A) 293T cells treated with Cdh1 (siCdh1), MOAP-1 (siMOAP-1), or control siRNA (siCtr) were treated with control or 100 μ M etoposide for 48 h. Bax activation was monitored by IP with 6a7 antibody from 293T cells followed by immunoblotting of total Bax (N20). Input lysates were immunoblotted with anti-actin and -Bax (N20) antibodies. (B) Cell lysates from A were immunoblotted with anti-Cdh1 or anti-MOAP-1 antibodies. (C) Control, Cdh1, and MOAP-1 knockdown PC3 cells were treated with 100 μ M etoposide for 48 h. The percentage of cells with sub-G1 DNA content was measured by PI staining and flow cytometry. (D) PC3 cells were processed as in C but treated with 20 μ M cisplatin. (C and D) The asterisks indicate that the difference between the two experiments is significant ($P < 0.05$), using an unpaired t test. The data shown represent three independent experiments. Error bars are SEM. (E) Cdh1 or MOAP-1 was knocked down using siRNA in PC3 cells, and samples were immunoblotted for Cdh1, MOAP-1, or actin. (F) Cells treated as in D were lysed, and lysates were immunoblotted to detect caspase-3 processing.

3. Ubiquitylation of p53 by the APC/C inhibitor TRIM39

This chapter is adapted from (Zhang et al., 2012a)

3.1 Summary

As described in previous chapter, TRIM39 is a RINGdomain-containing E3 ubiquitin ligase able to inhibit the anaphase-promoting complex (APC/C) directly. Through analysis of TRIM39 function in p53-positive and p53-negative cells, we have found, surprisingly, that p53-positive cells lacking TRIM39 could not traverse the G1/S transition. This effect did not result from disinhibition of the APC/C. Moreover, although TRIM39 loss inhibited etoposide-induced apoptosis in p53-negative cells, apoptosis was enhanced by TRIM39 knockdown in p53-positive cells. Furthermore, we show here that the TRIM39 can directly bind and ubiquitylate p53 in vitro and in vivo, leading to p53 degradation. Depletion of TRIM39 significantly increased p53 protein levels and cell growth retardation in multiple cell lines. We found that the relative importance of TRIM39 and the well-characterized p53-directed E3 ligase MDM2, varied between cell types. In cells that were relatively insensitive to the MDM2 inhibitor, nutlin-3a, apoptosis could be markedly enhanced by siRNA directed against TRIM39. As such, TRIM39 may serve as a potential therapeutic target in tumors with WT p53 when MDM2 inhibition is insufficient to elevate p53 levels.

3.2 Introduction

E3 ubiquitin ligases are essential for cell cycle transitions, controlling the abundance of many key cell cycle regulators such as Cyclin proteins and their inhibitors (Wickliffe et al., 2009). In addition, E3 ligases such as MDM2, a ligase that targets the p53 tumor suppressor, are known to control the cellular response to DNA damage including cell cycle arrest and the induction of apoptosis (Haupt et al., 1997; Honda et al., 1997; Kubbutat et al., 1997)

Recently, we reported that involvement of the TRIM39 E3 ubiquitin ligase in controlling DNA damage-induced apoptosis through inhibition of the APC/C, a multi-protein ubiquitin ligase that controls multiple cell cycle regulators including Cyclins, geminin, and others (Huang et al., 2012). Surprisingly, analysis of cell cycle progression in cells lacking TRIM39 now suggests that it also might be important for G1/S progression/initiation of DNA replication in some cell types, but that a target other than the APC/C was likely involved.

p53 is a sequence-specific transcription factor that controls a host of genes important for cell death and proliferation (Vousden and Lane, 2007). p53 protein is crucial for protecting cells in times of stress through a combination of cell cycle arrest, apoptosis, cellular senescence, DNA repair and autophagy (Lane, 1992; Vogelstein et al., 2000). A variety of stressors can engage the p53 pathway, and p53 mutations, often lying within the p53 DNA-binding domain or within its protein interaction domains are

present in greater than half of human cancer cases (Hainaut and Hollstein, 2000; Toledo and Wahl, 2006).

Given the centrality of p53 in the cellular stress response and the potential deleterious impact of alterations in p53 levels (eg. cell cycle arrest, cell death) under non-stressed conditions, levels of cellular p53 are tightly controlled via ubiquitin-mediated proteasomal degradation (Brooks and Gu, 2011; Kruse and Gu, 2009). The most intensively-studied p53-directed E3 ligase is MDM2, proven in many settings to promote p53 proteasomal degradation (Haupt et al., 1997; Honda et al., 1997; Kubbutat et al., 1997). Indeed, MDM2 inhibitors, such as nutlin-3a, are potential cancer therapeutics aimed at increasing p53 abundance to reduce proliferation and enhance tumor cell death/senescence (Shangary et al., 2008; Vassilev et al., 2004). Despite the importance of MDM2 in regulating p53 levels, p53 is susceptible to degradation in *MDM2*-deficient mice (Ringshausen et al., 2006), indicating the presence of other E3 ligases able to target p53. In this regard, several E3 ligases including Pirh2, COP1, ARF-BP1, CHIP, CARP1, CARP2 can promote p53 ubiquitylation and degradation under specific circumstances and/or in specific cell types (Brooks and Gu, 2011).

Here we report that the TRIM39 E3 ubiquitin ligase, previously identified as a regulator of the APC/C, has a dual role as a direct E3 ubiquitin ligase for p53. Accordingly, the G1/S arrest observed following depletion of TRIM39 was dependent on p53. The ability of TRIM39 to induce p53 ubiquitylation and degradation did not

depend on the APC/C and was also independent of MDM2. Accordingly, for several cell lines that were relatively insensitive to nutlin-3a, depletion of TRIM39 increased apoptotic cell death. This identification of a previously unknown negative regulator of p53 suggests that there are likely to be as-yet-unexplored strategies for elevating p53 for therapeutic benefit.

3.3 Materials and methods

3.3.1 Cell culture and synchronization

Cultured cells were maintained in the indicated media supplemented with 10% FBS: ARPE-19, human telomerase reverse transcriptase-immortalized retinal pigment epithelial cell line (hTERT-RPE), MCF-7, A549, H1299, U-87MG, T98G, JEG-3 (DMEM), 22Rv1, PC3 (RPMI 1640), U-2OS, and HCT116 (McCoy's 5A). Stable knockdown of p53 in hTERT-RPE was performed using the vector shp53-pLKO.1-puro (plasmid 19119; Addgene) (Godar et al., 2008). For nocodazole synchronization, cells were double thymidineblocked using 2.5 mM thymidine and released into medium containing 100 ng/mL nocodazole. To arrest cells at G1 phase by serum starvation, hTERT-RPE cells were maintained in serumfree media for 6 d before adding back normal media to commence the time course.

3.3.2 siRNA knockdown

Lipofectamine RNAiMax (Invitrogen) was used for siRNA transfections. The following siRNAs were used: tripartite motif 39 (TRIM39) siRNA (J-007028-07:

GCAACUAGGCAGUAUGGUG, J-007028-08: GAAGAAUGCUGCACCACUU, and SMARTpool L-007028-00; Dharmacon), APC11 siRNA (L006992- 00-0005; Dharmacon), p53 siRNA (TP53HSS186391; Invitrogen), murine double minute 2 (MDM2) siRNA (UUCCGAAGCUGGAAUCUGUGAGGUG), and control siRNA directed against firefly luciferase (CGUACGCGGAAUACUUCGA).

3.3.3 Plasmids and cell transfection

pcDNA3-TRIM39 and pMAL-c2x-TRIM39 were as previously described (Huang et al., 2012). pEGFP-C1 vector was from Clontech. Cells were transfected with XtremeGENE 9 (Roche) per the manufacturer's instructions.

3.3.4 Antibodies, western blotting, and immunoprecipitation

The following antibodies were used: anti-actin (sc-1616-R), anti-Cyclin B1 (sc-245), anti-geminin (sc-13015), anti-Cyclin A (sc-751), anti-p53 (sc-126), anti-MDM2 (sc-965), anti-I κ B- α (sc-371), anti-HA (sc-7392), anti-Myc (sc-40), anti-GFP (sc-9996), and anti-GST (sc-138) (all from Santa Cruz Biotechnology); anti-lamin B (ab16048) and anti-maltose binding protein (anti-MBP, ab9084) (both from Abcam); anti-p21 (556431; BD Pharmingen); and anti-Emi1 (37-6600; Invitrogen). For secondary antibodies, antimouse or anti-rabbit light chain-specific HRP antibodies (Jackson ImmunoResearch Laboratories) were used for Western blotting of coimmunoprecipitation. For TRIM39 endogenous coimmunoprecipitation, cells were cross-linked with DSP

(Dithiobis[succinimidyl propionate]) (22585; Thermo Scientific) as described (Zlatic et al., 2010).

3.3.5 Quantitative real-Time PCR analysis

Quantitative real-time PCR analysis was carried out as described (Zhang et al., 2011). All samples were amplified in triplicate, and every experiment was repeated at least two times. Relative gene expression was converted using the $2^{-\Delta\Delta C_t}$ method against the internal control GAPDH housekeeping gene. The following primers were used: APC11 (forward: CAG GAT GGC ATT TAA CGG ATG, reverse: AGA CTC TTC CCT AGA CAT CAG G), Bax (forward: AGT GTC TCA AGC GCA TCG, reverse: AAG TCC AAT GTC CAG CCC), Cyclin A (forward: CCAAGAGGACCA GGA GAA TAT C, reverse: CGC AGG CTG TTT ACT GTT TG), Cyclin B1 (forward: CTT TCG CCT GAG CCT ATT TTG, reverse: GTA TTT TGG TCT GAC TGC TTG C), Cyclin D1 (forward: CAT CTA CAC CGA CAA CTC CAT C, reverse: GTT CAA TGA AAT CGT GCG G.), Cyclin E (forward: GAT CTC TGT GTC CTG GAT GTT G, reverse: GTC TCT GTG GGT CTG TAT GTT G), Emi1 (forward: GTG TCT AAA GTG AGC ACA ACT TG, reverse: TTC TCT GGT TGA AGC ATG AGG), GAPDH (forward: ACA TCG CTC AGA CAC CAT G, reverse: ATG ACA AGC TTC CCG TTC TC), geminin (forward: TCC CAG TATGAA GCA GAA ACA AG, reverse: CAG GGCTGG AAG TTG TAG ATG), MDM2 (forward: AAG GAG AGC AAT TAG TGA GAC AG, reverse: TTA CAC CAG CAT CAA GAT CCG), p16 (forward: CAC CAG AGG CAG TAA CCATG, reverse: TGA TCT

AAG TTT CCC GAG GTT TC), p21 (forward: TGT CAC TGT CTT GTA CCC TTG, reverse: ACA CAA ACT GAG ACT AAG GCA G), p27 (forward: TCT GAG GAC ACG CAT TTG G, reverse: AAT TCG AGC TGT TTA CGT TTG AC), p53 (forward: TGC GTG TGG AGT ATT TGG ATG, reverse: AGG ACA GGC ACA AAC ACG), and retinoblastoma-associated protein (pRB) (forward: ACC AAT ACC TCACAT TCC TCG, reverse: GGT CGC TGT TAC ATA CCA TCT).

3.3.6 Cytosol and nuclei fractionation

Cytoplasmic and nuclear extracts were isolated by using the NE-PER Nuclear and Cytoplasmic Extraction Kit (Thermo Scientific) following the manufacturer's instructions.

3.3.7 Immunofluorescence and live cell imaging

Cells were cultured on glass coverslips in six-well plates. Cells were fixed with 4% (wt/vol) paraformaldehyde (PFA) prepared in PBS for 15 min and then permeabilized with PBS containing 0.5% (vol/vol) Triton X-100 for 5 min. Fixed cells were incubated with the appropriate primary antibodies in 5% nonfat dry milk, followed by incubation with the appropriate Alexa Fluor 488-conjugated secondary antibodies (Invitrogen) for 1 h and Hoechst 33258 (Molecular Probes) for 5 min to stain the nuclei. Fluorescent images were acquired using a Leica SP5 confocal scanning microscope. Images were analyzed using MetaMorph software (MDS Analytical Technologies). Timelapse phase-contrast images were collected every 10 min from cells

grown in glass-bottomed plates (MatTek) using a Zeiss Axio Observer Z1 microscope with a Photometrics Coolsnap ES2 camera.

3.3.8 In vitro ubiquitylation

Briefly, immunoprecipitated HA-p53 from 293T cells or 0.5 µg of GST-p53 (14-865; Millipore) was incubated with 1 µg of MBP-TRIM39 (1 h at 30 °C) with 50 ng of rabbit E1, 1 µg of UbcH5a, 10 µg of ubiquitin, and 3 mM ATP brought to 40 µL with buffer [25 mM Hepes (pH 7.5), 10 mM NaCl, 3 mM MgCl₂, 10 mM DTT, and 0.05% Triton X-100]. Reactions were stopped by adding SDS sample buffer, resolved by SDS/PAGE, and analyzed by immunoblotting.

3.3.9 In vivo ubiquitylation

HEK293 cells were transiently transfected with TRIM39, myc-p53, HA-ubiquitin, or GFP constructs as indicated. After 36 h, the transfected cells were treated with 20 µM MG132 for a further 8 h. Cell lysates were prepared in radioimmunoprecipitation assay lysis buffer with protease inhibitor mixture and MG132 (20 µM). Myc-p53 was immunoprecipitated from the cell extract using anti-Myc antibodies, and Western blotting was performed to detect p53 ubiquitylation.

3.3.10 Pull-down assays

Pull-down assays were performed as previously described (Huang et al., 2012). Briefly, 2 µg of MBP or MBP-TRIM39 was incubated with in vitro-translated HA-p53 or 2 µg of GST-p53 for 2.5 h at 4 °C. Amylose beads were added for another 30 min to pull

down MBP proteins and washed five times with immunoprecipitation buffer. Samples were analyzed by immunoblotting.

3.3.11 Growth curve and colony formation assay

For growth curve assays, hTERT-RPE cells were transfected with the indicated siRNAs; 48 h later, 1×10^5 cells were reseeded to six-well plates, and cell numbers were counted every day. For colony formation assay, 500 cells were seeded to 6-cm or 10-cm cell culture dishes 48 h after siRNA transfection. After 12 d, cells were fixed with 4% PFA for 5 min, followed by staining with 0.05% Crystal Violet for 30 min and washing two times with tap water. The dishes were scanned to count colonies.

3.3.12 Statistical analysis

All results are expressed as the mean \pm SD. Comparisons between two groups were made by an unpaired Student t test. A p value less than 0.05 was considered as statistically significant. All experiments were repeated at least three times to allow for appropriate statistical analyses.

3.3.13 Flow cytometry analysis

For the proliferation assay, the Click-iT EdU Alexa Fluor 488 Flow Cytometry Assay Kit (Invitrogen) was used to measure cell proliferation following the manufacturer's instructions. For cell cycle analysis, cells were fixed in 70% ethanol (vol/vol) (prechilled at -20°C) for at least 30 min and incubated in 15 $\mu\text{g/mL}$ RNase A (Roche) for 15 min, followed by incubation in 50 $\mu\text{g/mL}$ PI (Sigma) for 30 min. For the

annexin V/PI apoptosis assay, cells ($\sim 1 \times 10^5$) were collected and washed once with PBS, followed by incubation in annexin V (A13201; Invitrogen) solution [2.5 μ L of annexin V in 50 μ L of annexin V binding buffer (10 mM Hepes, 140 mM NaCl, 2.5 mM CaCl_2)] for 15 min and 300 μ L of 2 μ g/mL PI in annexin V binding buffer. Flow cytometry analysis was carried out with the use of a Becton–Dickinson FACScan apparatus and CellQuest software.

3.4 Results

3.4.1 Knockdown of TRIM39 induces G1/S arrest and inhibition of proliferation

Based on its ability to inhibit the APC/C, we had postulated that TRIM39 protein might be a component of the spindle assembly checkpoint that prevents APC/C activation when chromosomes are not properly aligned on the mitotic spindle. To assess this, we transfected cells with TRIM39-directed siRNA able to deplete greater than 90% of TRIM39 from hTERT-RPE cells (where we had seen a marked effect on APC/C-mediated protein degradation), synchronized these cells using double thymidine block, release into nocodazole to disrupt the spindle and monitored cells by time-lapse microscopy or analyzed their DNA content by flow cytometry (Fig. 3.1A and 3.1B). We were surprised to find that the great majority of cells lacking TRIM39 (93%) never entered mitosis (Fig. 3.1A and 3.1B). These data suggested that there might be another critical role for TRIM39 in promoting cell cycle progression. Indeed, proliferation of the RPE cells was markedly dampened by loss of TRIM39, as shown by both cell growth

curves and colony forming assays (Fig. 3.2A and 2 B). Moreover, EdU incorporation was significantly decreased after knockdown of TRIM39 (1.3% vs. control 25.9%, $p < 0.05$), demonstrating that the lack of TRIM39 prevented DNA replication (Fig. 3.2C and 3.1C). Together, these data suggested that TRIM39 has a role at the G1/S transition (under normal, non-stressed conditions), in addition to its role in controlling apoptosis in response to DNA damage. Indeed, cell death did not contribute to failure of TRIM39-deficient cells to proliferate as depletion of TRIM39 had little effect on basal rates of cell death in hTERT-RPE cells as determined by Annexin V/PI staining (Fig. 3.1D).

3.4.2 G1 arrest induced by depletion of TRIM39 is dependent on p53

The APC/C inhibitor Emi1 enables DNA synthesis by allowing accumulation of APC/C substrates such as Cyclin A and other factors required for the G1/S transition (Machida and Dutta, 2007; Miller et al., 2006; Reimann et al., 2001a; Reimann et al., 2001b). Thus, it remained possible that TRIM39 also controlled G1/S through its ability to modulate the APC/C. hTERT-RPE cells were serum starved to arrest at G0/G1 phase, then released into serum-containing medium. As shown in Fig. 3.3A, the levels of the APC/C substrates geminin and Cyclin A rapidly increased in a time dependent manner in control cells, whereas these increases were not observed in cells treated with TRIM39 siRNA. A similar decrease in Cyclin A and geminin accumulation could be seen upon loss of Emi1 (Fig. 3.3B). However, additional analysis revealed a fundamental difference between loss of Emi1 and TRIM39; proteasome inhibition by MG132 was able to restore

Cyclin A and geminin levels following knockdown of Emi1, while proteasome inhibition had a minimal effect on the levels of these proteins following TRIM39 knockdown (Fig. 3.3B). These data suggested that the effect of TRIM39 on Cyclin A and geminin expression was unlikely to be a result of TRIM39-mediated APC/C inhibition.

To address these issues further, we performed quantitative real-time PCR on control cells or cells treated with TRIM39 siRNA. As shown in Fig. 3.3C, there was a significant decrease in the levels of Cyclin A, geminin, and Emi1 mRNAs in the absence of TRIM39 (Fig. 3.3C). However, we observed a marked increase in the expression of mRNAs encoding p21 (but not p27 and p16), MDM2 and Bax after TRIM39 depletion. These genes are well known targets of the tumor suppressor p53, raising the possibility that p53 was, in some way, involved in the G1/S transition defect observed in cells lacking TRIM39. Therefore, we examined the effects of co-depletion of p53 and TRIM39. While the protein levels of p53 and p21 were increased coordinately with decreased Cyclin A and geminin proteins after depletion of TRIM39, control levels of these proteins was restored after co-depletion of TRIM39 and p53 (Fig. 3.3D). These data strongly suggested that the G1/S arrest resulting from TRIM39 depletion in RPE cells was exerted through p53. Indeed, knockdown of p53 largely (albeit not completely) restored EdU incorporation and DNA replication following knockdown of TRIM39 (Fig 3.3E, 3.4A, 3.4B and 3.4C). Extending these results to other cell types, and consistent with this idea, we found that depletion of TRIM39 decreased EdU incorporation in cells

with wild type p53 (A549, U-87 MG, 22Rv1, MCF7, HepG2, HCT116 P53+/+), but had either a minor or no effect on EdU incorporation in p53 mutant or null cells (H1299, T98G, PC3, HCT116 P53-/-) (Fig. 3F). Collectively, these data suggest that TRIM39 depletion can induce accumulation of p53 and its target, p21, resulting in a failure to traverse the G1/S transition.

3.4.3 TRIM39 interacts with p53 and regulates its stability

Given the effects of TRIM39 on p53 levels, we tested whether TRIM39 and p53 could interact. As shown in Fig. 3.5A, TRIM39 and p53 proteins were both predominantly nuclear and the nuclear fraction of p53 was dramatically increased in abundance following TRIM39 depletion (Fig. 3.5A, also shown in micrographs in Fig. 3.4D). In addition, we were able to co-immunoprecipitate p53 and TRIM39 with antisera directed against either protein (Fig. 3.5B, C, D, 3.6A, 3.6B). Moreover, we were able to demonstrate a direct interaction between recombinant TRIM39 and p53 proteins in vitro (Fig. 3.6C, 3.6D).

Since TRIM39 depletion increased p53 protein levels and we had previously characterized TRIM39 as an RING E3 ligase, we monitored the effect of TRIM39 on p53 protein stability and found, after depletion of TRIM39, that the half life of p53 was significantly increased (Fig. 3.7A, 3.8A, 3.8B). Importantly, although protein levels of p21 were also dramatically increased after depletion of TRIM39, the p21 half life was not changed, indicating that TRIM39 does not regulate protein levels of p21 directly (Fig.

3.7A, 3.8A, 3.8B.) Rather, it is likely that changes in p21 levels reflect changes in its transcriptional activator, p53. TRIM39 overexpression decreased levels of p53; this decrease was dependent on TRIM39 E3 ligase activity as overexpression of ligase-deficient RING domain mutants could not promote p53 degradation, despite their ability to bind p53 (Fig 3.7B and 3.5D). The proteasome inhibitor MG132 allowed preservation of p53 protein in the face of wild-type TRIM39 overexpression, consistent with a role for TRIM39 in p53 degradation (Fig. 3.9A). In addition, the half life of exogenously expressed p53 was significantly decreased upon coexpression of wild type TRIM39 and was actually increased with coexpression of the ligase-deficient RING domain mutant, likely acting in a dominant negative fashion to impede endogenous Trim 39 (Fig. 3.8C).

Furthermore, ectopic expression of wild type TRIM39, but not the RING mutant protein, increased p53 ubiquitylation, consistent with the possibility that TRIM39 acts as a p53-directed E3 ubiquitin ligase (Fig. 3.9B). In support of this idea, TRIM39 was able to directly ubiquitylate p53 in vitro (Fig. 3.7C, 3.9C). Given that we had previously reported that TRIM39 could regulate APC/C activity, we speculated that there might be an unknown connection between the APC/C and p53 stability. However, depletion of Cdh1, the activator of APC/C in interphase, or APC11, a core subunit of the APC/C, had no effect on the ability of TRIM39 loss to elevate p53 levels (Fig. 3.11A, B), strongly suggesting that TRIM39 did not affect p53 levels by modulating the APC/C.

3.4.4 TRIM39 can synergize with MDM2 to affect cell growth and apoptosis

There are a number of E3 ubiquitin ligases that have been reported to act on p53, most prominently MDM2 (Haupt et al., 1997; Honda et al., 1997; Kubbutat et al., 1997). We wished to determine whether the regulation of p53 stability by TRIM39 was dependent upon MDM2 and thus we transfected H1299 cells lacking p53 with control or MDM2 siRNA, and examined the effects of TRIM39 on the stability of co-transfected p53. As shown in Fig. 3.10A, TRIM39 expression was able to induce p53 degradation even in the absence of MDM2. We also did not observe any direct effects of TRIM39 on MDM2 (or vice versa). Although TRIM39 knock-down was able to elevate MDM2 levels (Fig. 3.12A), this was an indirect effect resulting from stabilization of p53 (and therefore, enhanced MDM2 transcription) because knock-down of TRIM39 in H1299 cells lacking p53 had no effect on MDM2 levels (Fig. 3.12B). Moreover, it appears that TRIM39 and MDM2 bind to different domains of p53 as p53 mutants (L14Q/F19S, L22Q/W23S) that were unable to bind MDM2 bound as well to TRIM39 as the wild type p53 (Fig. 3.13A) (Lin et al., 1994).

To assess the relative contributions of TRIM39 and MDM2 in controlling cell cycle progression across a range of p53 wild type cell types, we knocked down TRIM39, MDM2, or both and measured EdU incorporation as well as protein levels of p53 and p21. As shown in Fig 3.10B and 3.12A, MDM2 and TRIM39 knockdown had variable impact on cell cycle progression in a variety of different cell types, with both ligases

contributing to the overall p53 and p21 levels as well as the ability to undergo DNA replication. That said, in cells where another p53-directed negative regulator (eg. MDMX in JEG-3 cells) is known to play a dominant role, neither knock-down of TRIM39 nor MDM2 (or both) had a significant impact on EdU incorporation (Fig. 3.13B).

MDM2 has been considered a promising target for cancer chemotherapy in that elevating levels of p53 in p53-positive tumors has the potential to induce cell cycle arrest and/or apoptosis. A number of MDM2 inhibitors have been developed, the most well known of which is nutlin-3a, a compound that interferes with the binding of p53 to MDM2 (Shangary et al., 2008; Vassilev et al., 2004). In contrast, we found that nutlin-3a was unable to disrupt the interaction of TRIM39 with p53 (Fig. 3.13C; compare lanes 2 and 5). Given the variable relative importance of MDM2 in different cell types, these data suggest that nutlin-3a might have only partial effects in cells expressing both MDM2 and TRIM39. Therefore, the utility of nutlin-3a as a therapeutic agent is likely to be restricted by the population of E3 ligases other than MDM2 (in this case, TRIM39) which contribute to p53 stability in a given cell type, particularly if those ligases, like TRIM39, are refractory to inhibition by nutlin-3a. Given this, we hypothesized that co-inhibition of TRIM39 would increase the efficacy of nutlin-3a in killing p53-positive cells. Consistent with this idea, knock down of TRIM39 enhanced cell killing by nutlin-3a in p53 wild type cells expressing both ligases, even in cells that were relatively refractory to nutlin-3a, such as MCF-7 breast cancer cells and A549 lung cancer cells

(Fig. 3.10C and D). Importantly, nutlin-3a, either alone, or in combination with TRIM39 knockdown, did not kill cancer cells in the absence of p53 (Fig. 3.10 D) (H1299, p53 null cancer cell).

We and others have reported that TRIM39 helps to induce apoptosis following DNA damage, due to its ability to elevate levels of the Bax activator MOAP-1 (by inhibiting MOAP-1 degradation by the APC/C). However, based on the data shown here, it seems that TRIM39 can also be considered anti-apoptotic, as its loss can promote stabilization of p53 and cell death. To further evaluate this issue, we knocked down TRIM39 in p53 wild type cell (A549) or p53 null cell (H1299) and treated with etoposide. As shown in Fig. 3.13D, knockdown of TRIM39 in the presence of functional p53 enhanced etoposide-induced cell death. In contrast, depletion of TRIM39 in cells lacking p53, dampened etoposide-induced apoptosis, likely at least in part, due to the effects of TRIM39 on the APC/C and its substrate, MOAP-1, as discussed further below.

3.5 Discussion

3.5.1 TRIM39 as a novel E3 ligase of p53

p53 is a central regulator of cell death, proliferation, and senescence and enables the cell to withstand a wide variety of stressors (Brown et al., 2009; Kruse and Gu, 2009; Wickliffe et al., 2009). For this reason, p53 mutation or deficiency contributes to a significant fraction of human tumors. Although p53 functions are required in the face of cellular stress or damage, normal proliferation of the unperturbed cell requires that

intracellular levels of p53 be restrained. Accordingly, a complex network of post-translational modifications (acetylation, methylation, phosphorylation, etc) fine tunes p53's half life, rendering it more or less susceptible to the action of E3 ubiquitin ligases (Kruse and Gu, 2009; Toledo and Wahl, 2006).

Although the E3 ubiquitin ligase MDM2 is thought to be the primary determinant of p53 ubiquitylation and stability in a variety of cell types, it is clearly not the only relevant p53-directed ligase (Camus et al., 2003; Ringshausen et al., 2006). Aside from the observation that p53 can be degraded in MDM2-deficient mice, mutation of the known MDM2-targeted lysines on p53 does not prevent p53 ubiquitylation and degradation in cells (Camus et al., 2003). Moreover, several other E3 ligases, including another TRIM family member, TRIM24, are capable of promoting p53 degradation (Allton et al., 2009). An additional level of complexity stems from the ability of several of the p53-directed ligases to control each other's stability, potentially through direct interaction (Wang et al., 2011).

In this study, we identified TRIM39 as a novel E3 ligase directly regulating p53 stability, and compared the differential effects of TRIM39 and MDM2 knock-down on cell proliferation, p53 stability and p21 induction in multiple cell lines. Interestingly, we found that the effect of these ligases varied in different cell types. For example, it seems that TRIM39 contributes more significantly than MDM2 to cell growth and p53 stability in hTERT-RPE cells. This may be of potential significance for diseases of the retina, for

example, macular degeneration, where p53 is important for controlling cell death in response to oxidative stress (Vuong et al., 2012).

3.5.2 TRIM39 inhibition could be used for cancer treatment

For tumors with wild type p53, numerous pharmacological approaches have been taken to up-regulate or stabilize p53 protein (Lane et al., 2010; Vassilev, 2007). Small molecules such as nutlin-3a and MI-219 are able to disrupt MDM2-p53 interactions, thereby preventing MDM2-mediated p53 ubiquitination (Shangary et al., 2008; Vassilev, 2007; Vassilev et al., 2004). While this approach to induce p53-mediated cell cycle arrest/apoptosis has been successful in many cancer cell lines and xenograft models (and is under investigation for treating human cancers), significant expression of other E3 ligases, such as TRIM39 would be expected to diminish the effects of nutlin-3a and similar molecules, unless the interaction of p53 with these other ligases was also disrupted. For example, nutlin-3a is already known to be ineffective in cells that express high levels of MDMX, another known p53-directed ligase (Hu et al., 2006; Patton et al., 2006; Wade et al., 2006). Given this, effective elevation of p53 in tumors may well require a cocktail of E3 ligase inhibitors tailored to the population of p53-directed ligases present in an individual tumor. Indeed, novel inhibitors that target both MDM2 and MDMX can impede growth and induce apoptosis in cells with high levels of both MDM2 and MDMX (Bernal et al., 2010; Lane et al., 2010; Reed et al., 2010).

In this study, we found that combining nutlin-3a with knockdown of TRIM39 was effective in triggering apoptosis in cells that were otherwise nutlin-3a insensitive (eg. MCF7 and A549.) However, this combination was not effective in JEG-3 cells, consistent with previous reports documenting high levels of MDMX expression in these cells.

Many of the E3 ligases that target p53 are also transcriptional targets of p53 (Brooks and Gu, 2011). Thus, while E3 ligase inhibition may elevate p53, p53 in turn can promote transcriptional up-regulation of the ligases, potentially overcoming the effect of the inhibitors. TRIM39 does not appear to be regulated by p53, as depletion of MDM2 significantly increases the levels of p53 without affecting the levels of TRIM39. This might make TRIM39 a promising therapeutic target, but we would note that depletion of TRIM39 significantly increases protein levels of both p53 and MDM2; induced MDM2 inhibition might therefore counter the effects of TRIM39 inhibition. These observations again support the notion of an inhibitor cocktail as perhaps the most effective approach to elevating p53 levels.

3.5.3 p53 status determines the outcome of TRIM39 activity

The identification of TRIM39 as a p53-directed ligase raises the question of how this activity may be related to its previously-described role as an APC/C inhibitor. It does not appear that the effects of TRIM39 on p53 are exerted through the APC/C nor do effects on the APC/C require p53 (as TRIM39 can inhibit the APC/C in p53-null cells).

Although TRIM39 E3 ligase activity is required to inhibit the APC/C, and TRIM39 can inhibit the purified APC/C in vitro, the direct target of TRIM39 in the APC/C has not yet been identified. It is possible that identification of determinants of p53 recognition/binding by TRIM39 may shed light on the direct interactors of TRIM39 within the APC/C. DNA damage-induced activation of Trim 39 appears to have different consequences depending on the p53 status of the cell. In the absence of p53, the Trim 39-mediated inhibition of the APC/C results in stabilization of MOAP-1, promoting Bax activation and apoptosis. Additionally, while the ability of TRIM39 to inhibit the APC/C seems to be activated by DNA damage, TRIM39 knockdown can affect p53 levels even in the unperturbed cell cycle. Taken together, these data place TRIM39 as a regulator of several key processes in the proliferative cycle. Additional experiments will determine whether these functions are entirely independent and whether they can be manipulated to therapeutic advantage in the setting of cancer, eye disease, or other pathologies involving mis-regulation of p53.

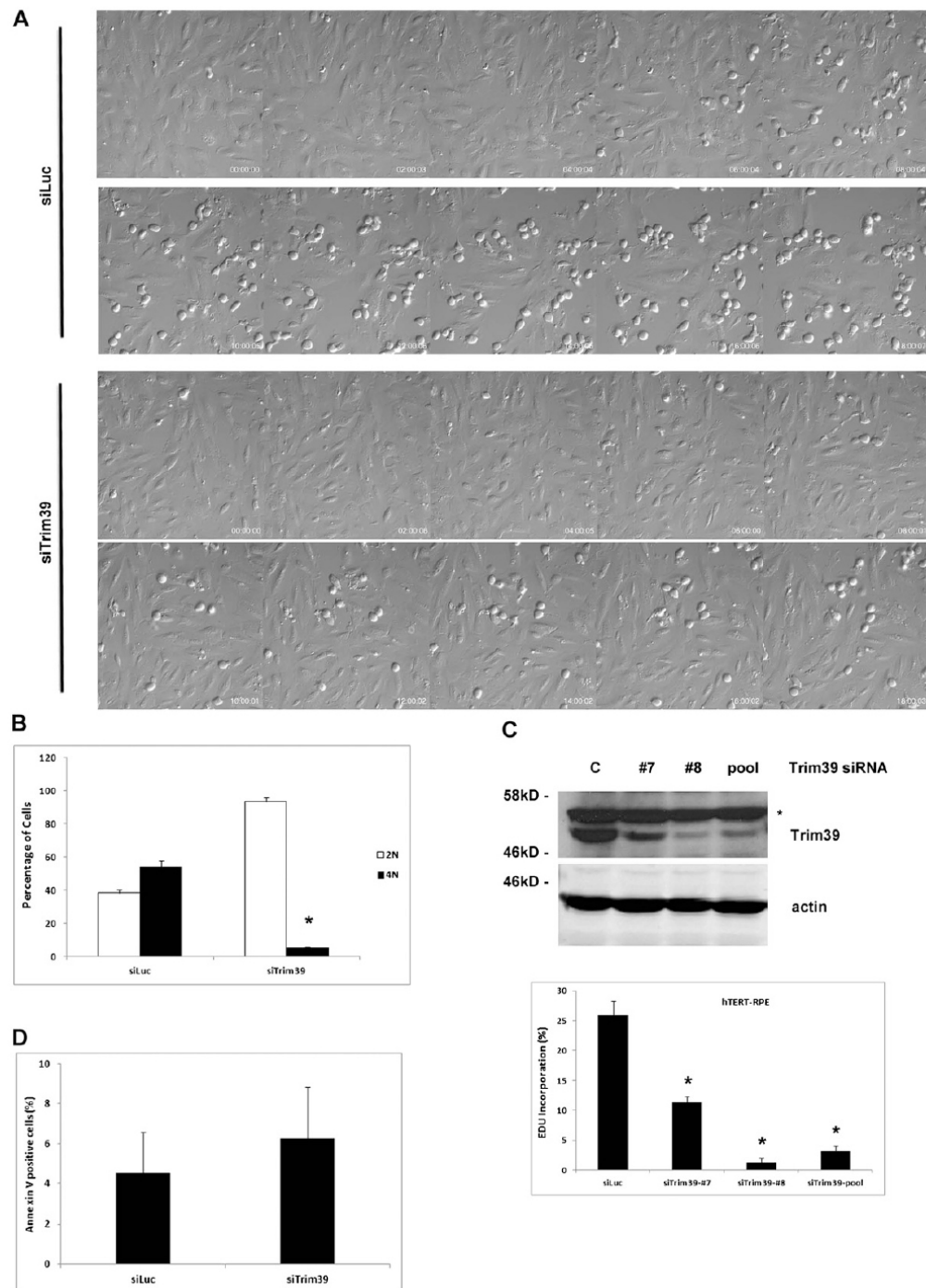


Figure 3. 1: Depletion of TRIM39 decreases cell proliferation.

Figure 3.1: (A) ARPE-19 cells were transfected with control or TRIM39 siRNA and released into nocodazole after a double thymidine block. Photomicrographs were taken every 10 min by time lapse for 24 h, and representative micrographs were selected for time points at 2-h intervals. Luc, luciferase. (Magnification: 165 \times .) (B) hTERT-RPE cells were transfected with control or TRIM39 siRNA and released into nocodazole for 16 h after the double thymidine block. Cells were collected and analyzed by flow cytometry. * $P < 0.05$ vs. control. (C) hTERT-RPE cells were transfected with control (C) or different TRIM39 siRNAs; the effect on TRIM39 protein levels was detected by Western blotting, and cell proliferation was detected by EdU (5-ethynyl-2-deoxyuridine) incorporation. * $P < 0.05$ vs. control. (D) hTERT-RPE cells were transfected with control or TRIM39 siRNA, and apoptosis was detected by annexin V and propidium iodide (PI) staining followed by flow cytometry.

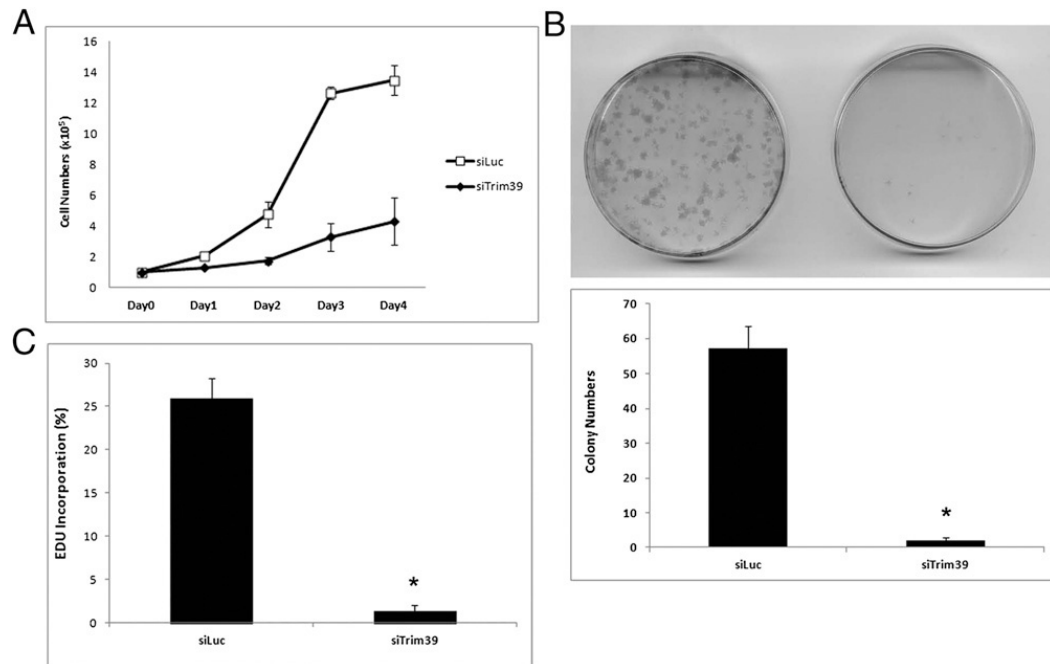


Figure 3. 2: TRIM39 depletion induces cell growth retardation.

Figure 3.2: A) hTERT-RPE cells were transfected with control or TRIM39 siRNA, and 1×10^5 cells were seeded in six-well plates 2 d after siRNA transfection. Cells were counted each day for 4 d. Luc, luciferase (B) Colony assay. Two days after siRNA transfection, 500 cells were seeded in 60-mm dishes and cultured for 12 d, and the number of colonies was counted. * $P < 0.05$ vs. control. (C) hTERT-RPE cells were transfected with control or TRIM39 siRNA, and DNA replication was monitored by EdU incorporation. * $P < 0.05$ vs. control.

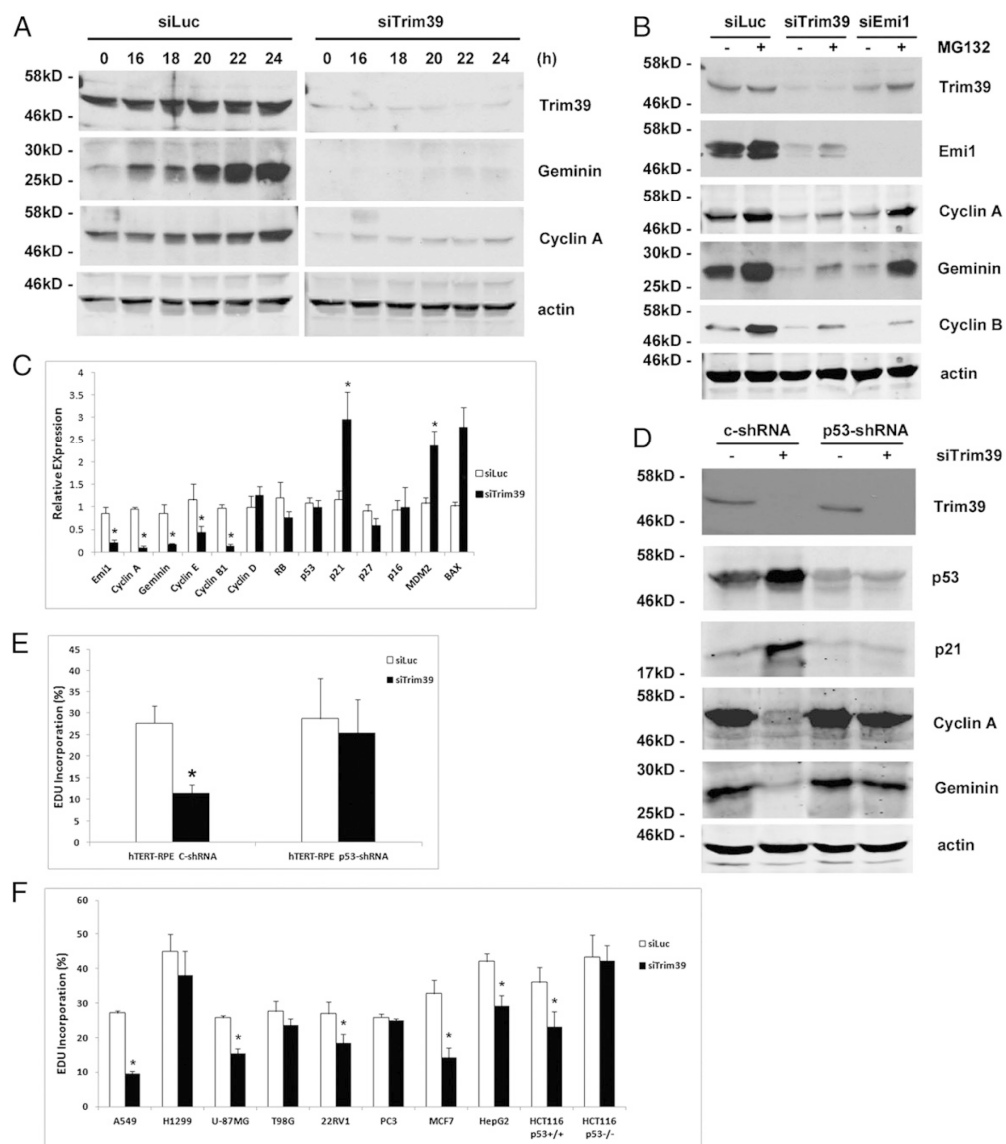


Figure 3. 3: Cell growth inhibition induced by TRIM39 depletion is p53-dependent.

Figure 3.3: (A) hTERT-RPE cells were arrested at G1 phase by serum starvation, and cells were harvested at different time points after adding back media with 10% FBS. The protein levels were detected by Western blotting with the indicated antibodies. Luc, luciferase. (B) Cells were transfected with control, TRIM39, or Emi1 siRNA; 48 h later, cells were treated with MG132 for a further 8 h. Western blotting was carried out to detect the protein level with the indicated antibodies. (C) Real-time PCR was carried out to detect the gene expression in control or TRIM39 knockdown cells. *P < 0.01 vs. control. (D) Control-shRNA or p53-shRNA cells were transfected with control or TRIM39 siRNA, and the protein levels were detected by Western blotting with the indicated antibodies. (E) Control-shRNA (c-shRNA) or p53-shRNA cells were transfected with control or TRIM39 siRNA, and cell proliferation was measured by EdU incorporation. *P < 0.05 vs. control. (F) Panel of cell lines with different p53 background was transfected with control or TRIM39 siRNA, and cell proliferation was detected by EdU incorporation. *P < 0.05 vs. control.

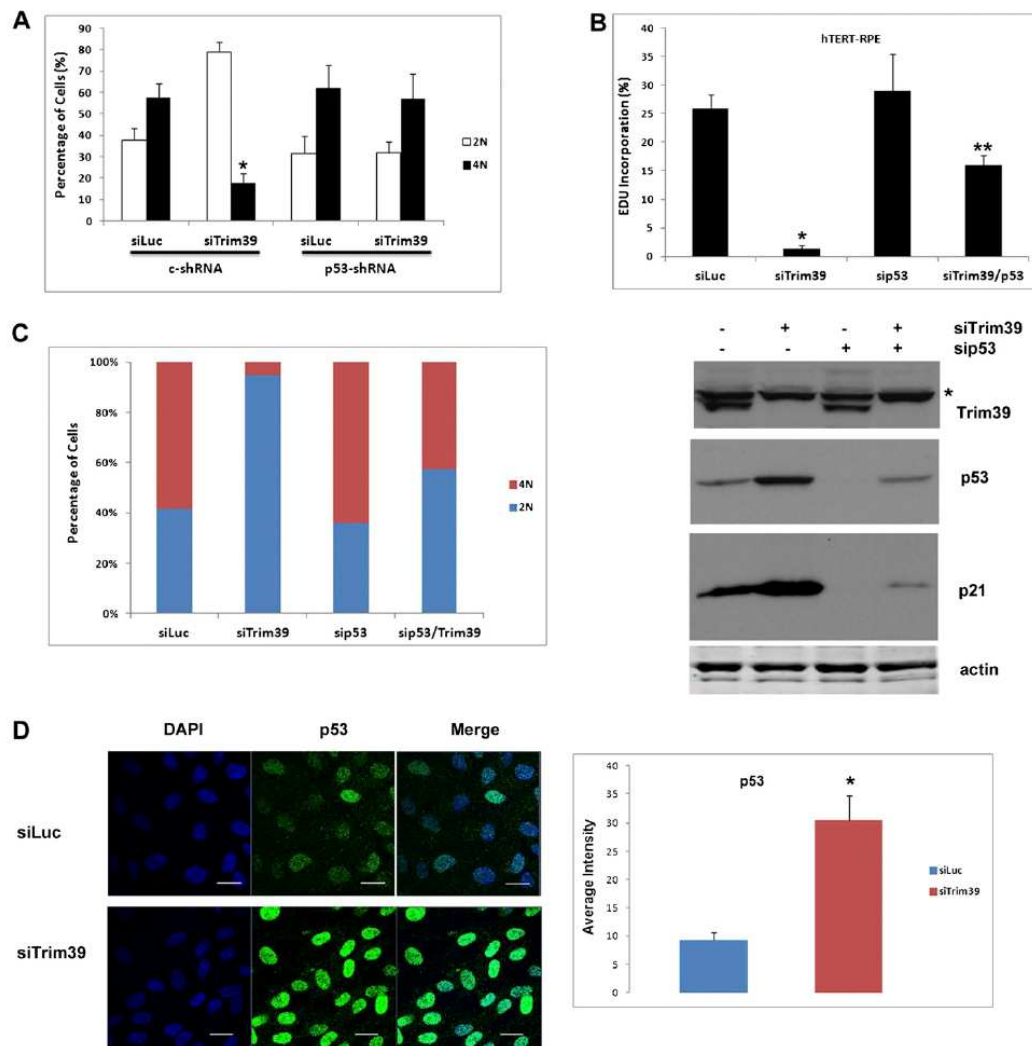


Figure 3. 4: Inhibition of cell growth induced by Trim 39 depletion is mediated by regulation of p53 stability.

Figure 3.4: (A) Control-shRNA (c-shRNA) or p53-shRNA hTERT-RPE cells were transfected with control or TRIM39 siRNA; 48 h later, cells were treated with nocodazole for 18 h. Cells were harvested, and cell cycle profiles were determined by flow cytometry. *P < 0.05 vs. control. Luc, luciferase. (B and C) hTERT-RPE cells were transfected with control, TRIM39, or p53 siRNA as indicated; cell proliferation was detected by EdU (5-ethynyl-2'-deoxyuridine) incorporation, and protein levels were measured by Western blotting (*P < 0.05 vs. control; **P < 0.05 vs. siTRIM39) (B), and cell cycle profiles were determined by flow cytometry (C). (D) hTERT-RPE cells were transfected with control or TRIM39 siRNA, and the protein levels of p53 were detected by immunostaining. (Scale bar, 20 μ m.) *P < 0.05 vs. control.

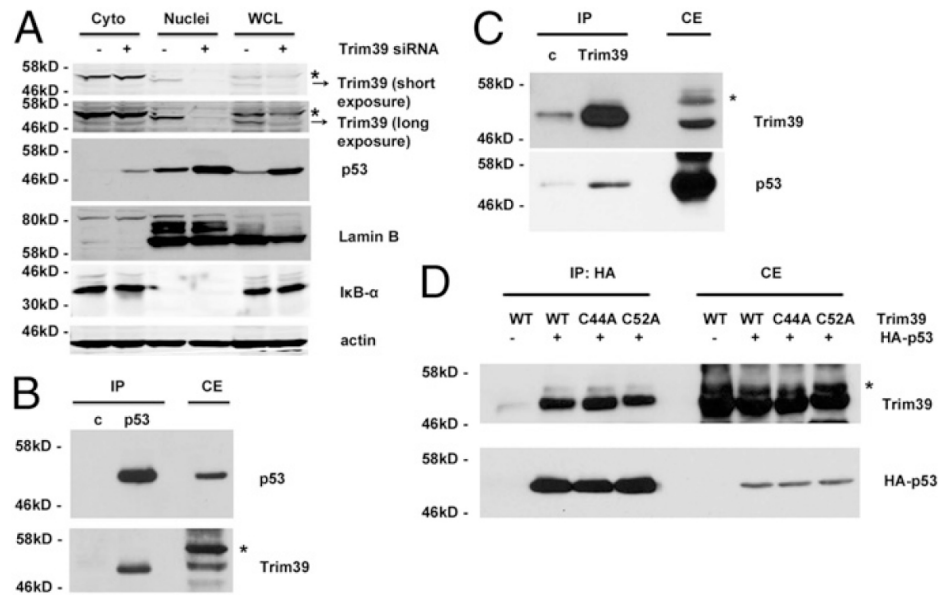


Figure 3. 5: TRIM39 localizes to the nucleus and interacts with p53.

Figure 3.5: (A) hTERT-RPE cells were transfected with control or TRIM39 siRNA, and cytoplasmic (Cyto) and nuclear extracts (Nuclei) were fractionated and analyzed by Western blotting with the indicated antibodies. The asterisk indicates nonspecific signal. (B and C) hTERT-RPE cells were cross-linked with DSP (Dithiobis[succinimidyl propionate]), and immunoprecipitation was carried out to detect the interaction between TRIM39 and p53. The asterisk indicates nonspecific signal. (D) 293T cells were transfected with HA-p53, TRIM39 WT, C44A, or C52A, respectively, as indicated. HA-p53 was immunoprecipitated with anti- HA antibody, and Western blotting was carried out to detect the interaction between TRIM39 and HA-p53. The asterisk indicates nonspecific signal. c, control; CE, cell extract; IP, immunoprecipitation; WCL, whole cell lysate.

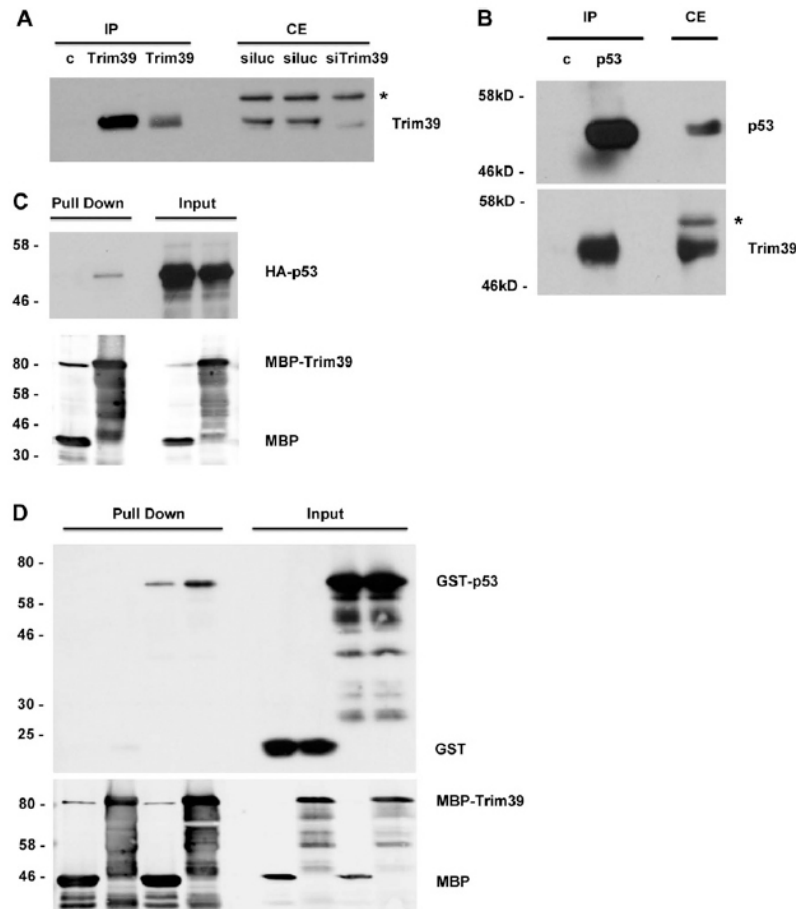


Figure 3. 6: TRIM39 interacts with p53 in vitro and in intact cells.

Figure 3.6: (A) hTERT-RPE cells were transfected with control (c) or TRIM39 siRNA; 48 h later, TRIM39 was immunoprecipitated with anti-TRIM39 antibody and detected by Western blotting. CE, cell extract; IP, immunoprecipitation. (B) MCF7 cells were cross-linked with DSP (Dithiobis[succinimidyl propionate]), and immunoprecipitation was carried out to detect the interaction between TRIM39 and p53. (C and D) Two micrograms of MBP or MBP-TRIM39 was incubated with in vitro-translated p53 (C) or 2 μ g of GST or GST-p53 (D) for 2 h at 4 $^{\circ}$ C, and amylose beads were added for a further 30 min. Beads were washed five times with immunoprecipitation lysis buffer, and protein was detected by Western blotting.

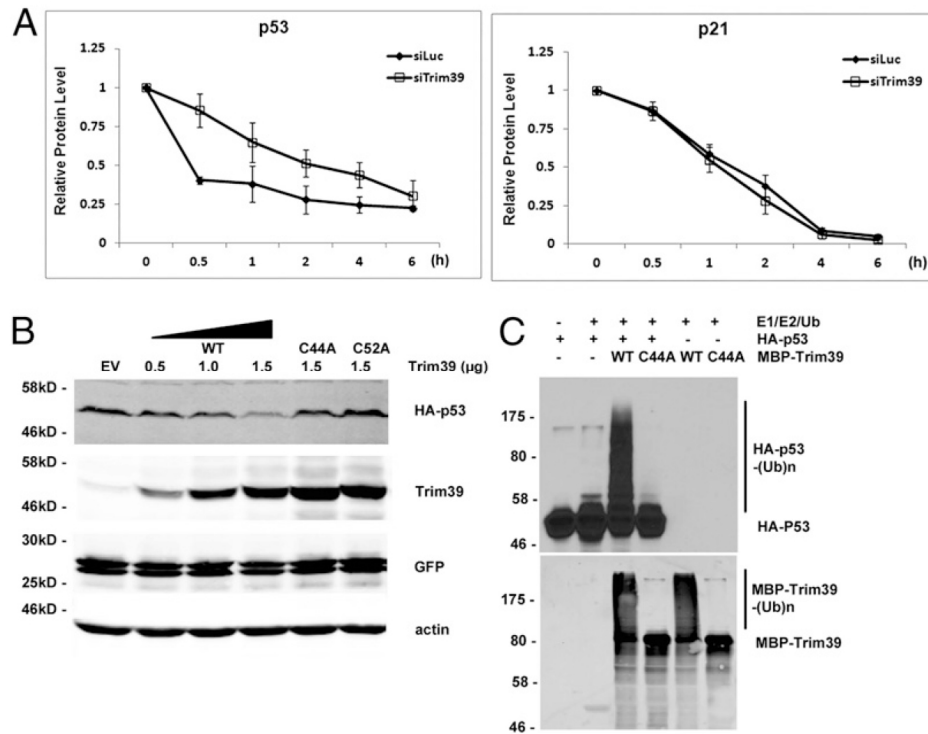


Figure 3. 7: TRIM39 negatively regulates p53 levels.

Figure 3.7: (A) hTERT-RPE cells were transfected with control or TRIM39 siRNA, and cells were treated with cycloheximide for the time courses indicated. The protein levels of p53 and p21 were detected by Western blotting, quantified using Odyssey software, and plotted against time to determine protein half-lives. Luc, luciferase. (B) H1299 cells were transfected with 0.2 μg of HA-p53, 0.1 μg of pEGFP-C1, and different amounts of TRIM39 WT or RING mutant-encoding plasmids as indicated. Cells were harvested 48 h after transfection, and protein levels were detected by Western blotting with the indicated antibodies. (C) In vitro ubiquitylation of p53 by TRIM39. 293T cells were transfected with HA-p53, and HA-p53 was immunoprecipitated and mixed with myelin basic protein (MBP)-TRIM39 and E1/E2/ubiquitin as indicated for 1 h at 30 °C. Reactions were stopped by adding sample buffer, and Western blotting was carried out to detect the ubiquitylation of p53.

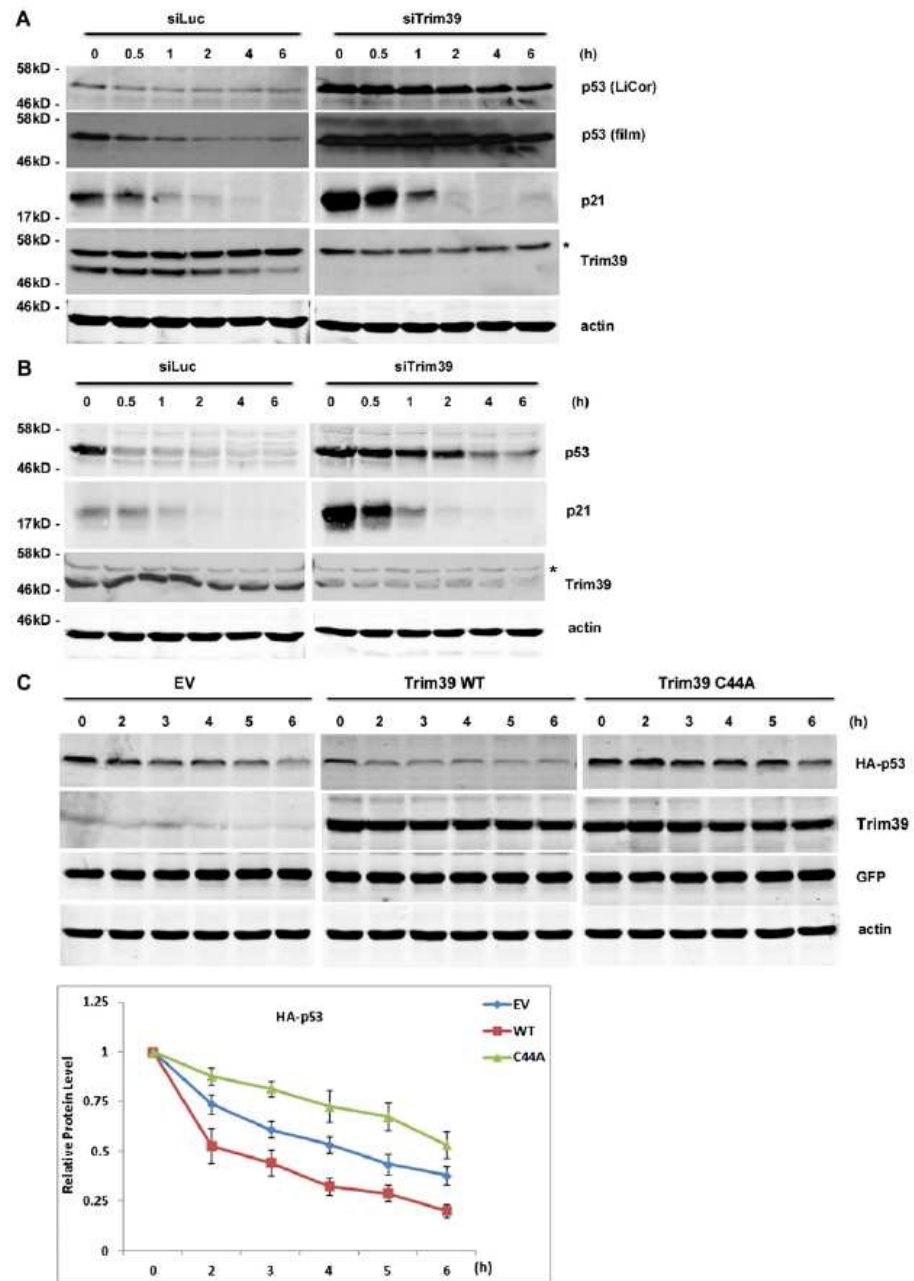


Figure 3. 8: TRIM39 negatively regulates the half-life of p53.

Figure 3.8: (A and B) hTERT-RPE cells were transfected with control or TRIM39 siRNA, and cells were treated with cycloheximide for the times indicated. The protein levels were detected by Western blotting. Luc, luciferase. (C) H1299 cells were transfected with 0.2 μ g HAp53, 0.1 μ g pEGFP-C1, and 1.5 μ g of TRIM39 WT or RING mutant-encoding plasmids as indicated. Forty-eight hours later, cells were treated with cycloheximide for the times indicated. The protein levels of HA-p53 were detected by Western blotting, quantified using LI-COR Biosciences Odyssey software, and plotted against time to determine protein half-lives. EV, empty vector.

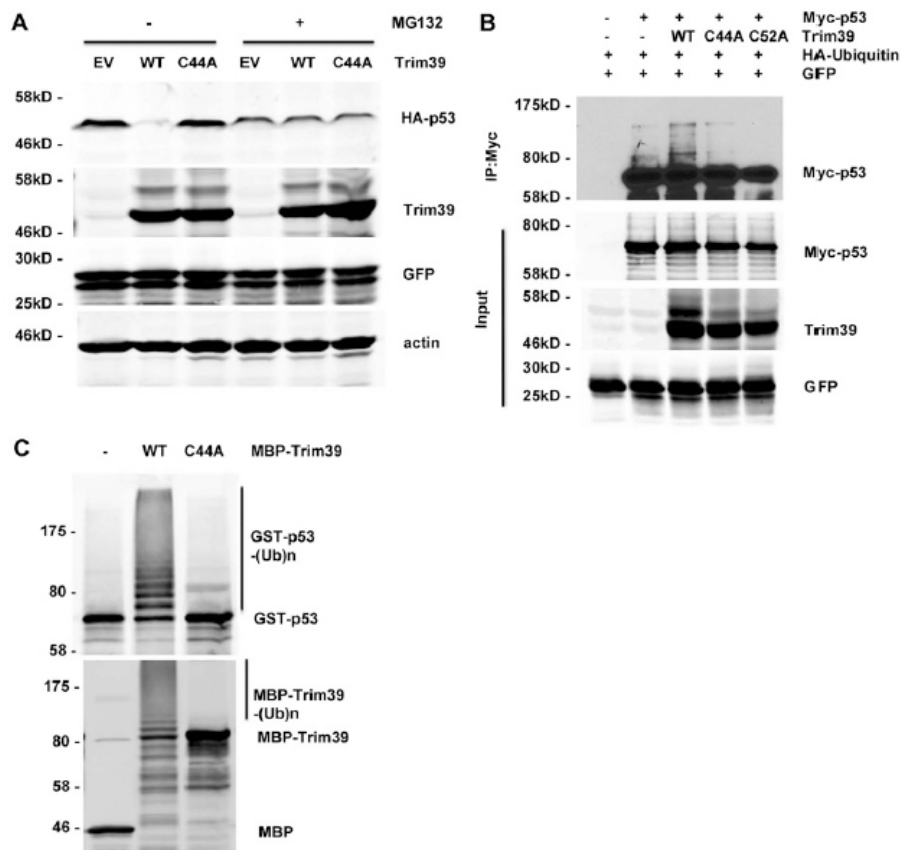


Figure 3. 9: TRIM39 can ubiquitylate p53 and regulate its stability.

Figure 3.9: (A) H1299 cells were transfected with HA-p53, pEGFP-C1, and TRIM39 WT or RING mutants, and MG132 was added for a further 8 h before harvesting the cells. Cells were harvested, and protein levels were detected by Western blotting. EV, empty vector. (B) In vivo ubiquitylation of p53 by TRIM39. 293T cells were transfected with HA-ubiquitin, myc-p53, pEGFP-C1, TRIM39 WT, or RING mutant as indicated. Cells were treated for a further 8 h with MG132 before harvesting. Cells were lysed, Myc-p53 was subjected to immunoprecipitation (IP) with anti-Myc antibody, and Western blotting was carried out to detect the ubiquitylation of p53 by TRIM39. (C) In vitro ubiquitylation of p53 by TRIM39. GST-p53 was mixed with MBP or MBP-TRIM39 and E1/E2/ubiquitin as indicated for 1 h at 30 °C. Reactions were stopped by adding sample buffer, and Western blotting was carried out to detect the ubiquitylation of p53.

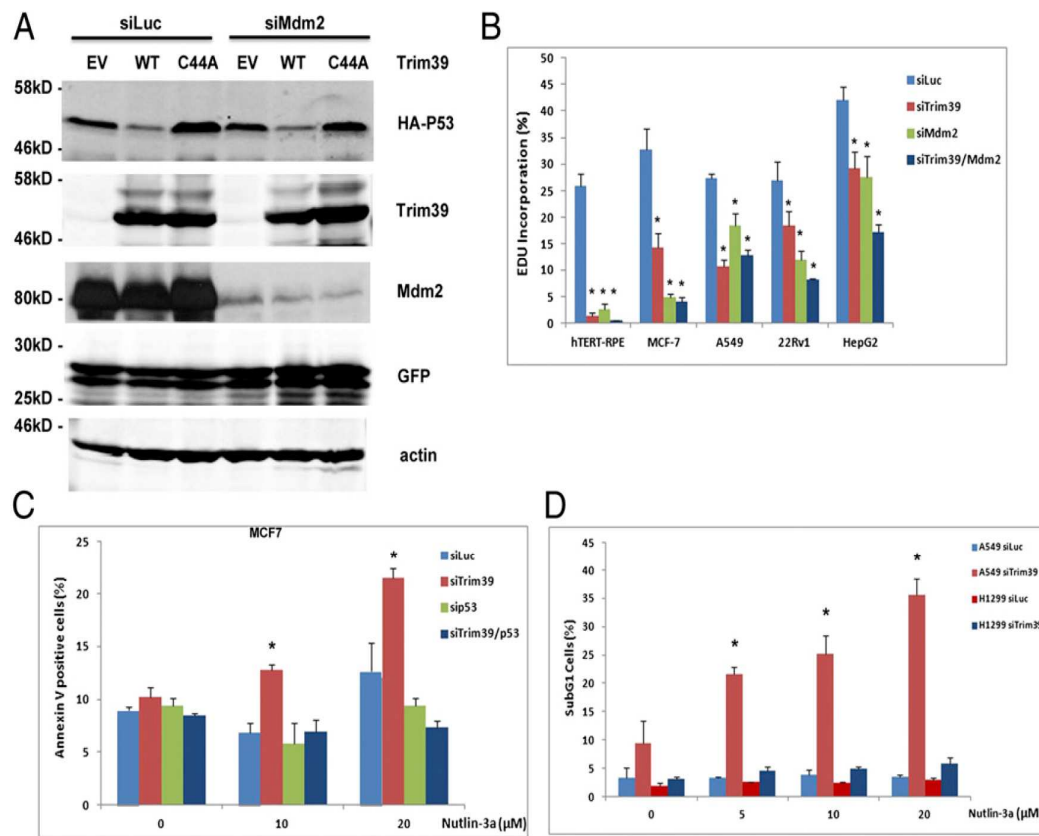


Figure 3. 10: Regulation of p53 stability by TRIM39 is independent of MDM2.

Figure 3.10: (A) H1299 cells were transfected with control or MDM2 siRNA first, and cells were then transfected with HA-p53, TRIM39 WT or RING mutants, and pEGFP-C1 as indicated 8 h after siRNA transfection. Cells were harvested 48 h later, and protein levels were detected by Western blotting with the indicated antibodies. Luc, luciferase; EV, empty vector. (B) hTERT-RPE, MCF7, A549, 22Rv1, or HepG2 cells were transfected with control, TRIM39, or MDM2 siRNA as indicated. Forty-eight hours later, cell proliferation was monitored by EdU incorporation. *P < 0.05 vs. control. (C) MCF7 cells were transfected with control, TRIM39, or p53 siRNA as indicated; 48 h later, cells were treated with nutlin-3a for a further 48 h. Cells were harvested and stained with annexin V and PI, and apoptosis was detected by flow cytometry. *P < 0.05 vs. control. (D) A549 or H1299 cells were transfected with control or TRIM39 siRNA, and 48 h later, cells were treated with nutlin-3a for a further 48 h. Apoptosis (as indicated by the presence of a sub- G1 population) was detected by flow cytometry. *P < 0.05 vs. control.

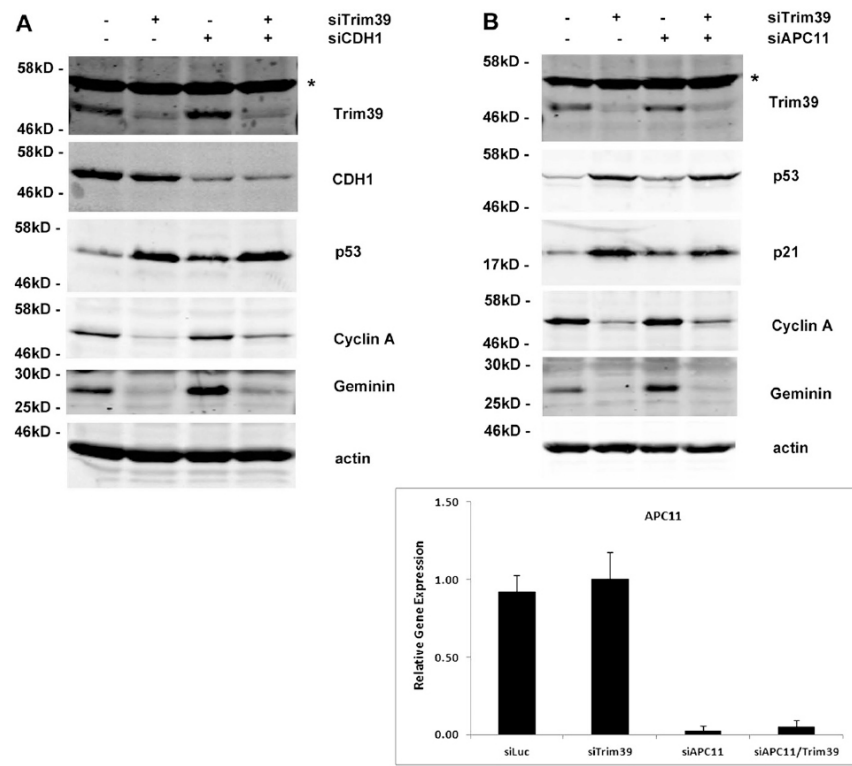


Figure 3. 11: Anaphase promoting complex (APC/C) is not involved in the regulation of p53 by TRIM39.

Figure 3.11: (A) hTERT-RPE cells were transfected with control, TRIM39, or CDH1 siRNA, and cells were harvested 48 h later. Protein levels in cell lysates were monitored by Western blotting with the indicated antibodies. (B) hTERT-RPE cells were transfected with control, TRIM39, or APC11 siRNA. Cells were harvested 48 h later, and protein levels were monitored by Western blotting with the indicated antibodies. The knockdown efficiency of siAPC11 was detected by quantitative PCR. Luc, luciferase.

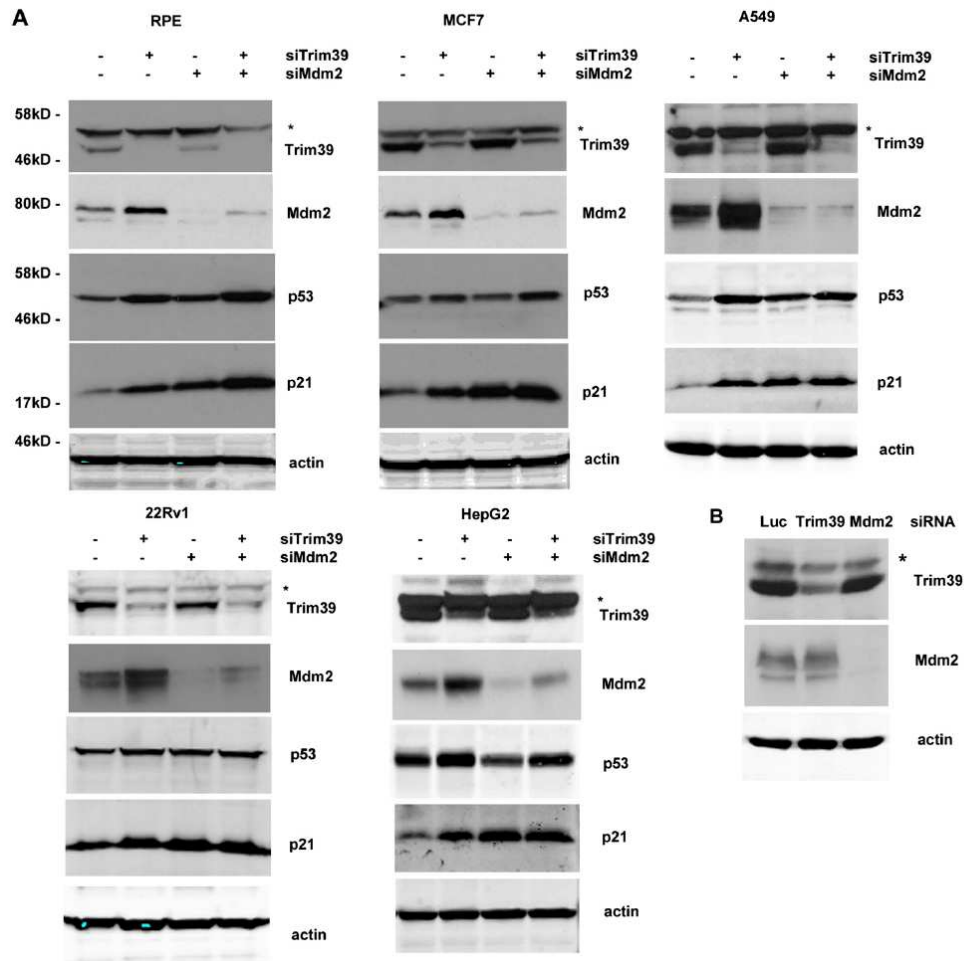


Figure 3. 12: TRIM39 and MDM2 act independently.

Figure 3.12: (A) hTERT-RPE (RPE), MCF7, A549, 22Rv1, or HepG2 cells were transfected with control, TRIM39, or MDM2 siRNA as indicated. Forty-eight hours later, protein levels were detected by Western blotting. *, nonspecific signal. (B) H1299 cells were transfected with control, TRIM39, or MDM2 siRNA. Forty-eight hours later, cells were harvested and Western blotting was carried out to detect protein levels. Luc, luciferase. *, nonspecific signal.

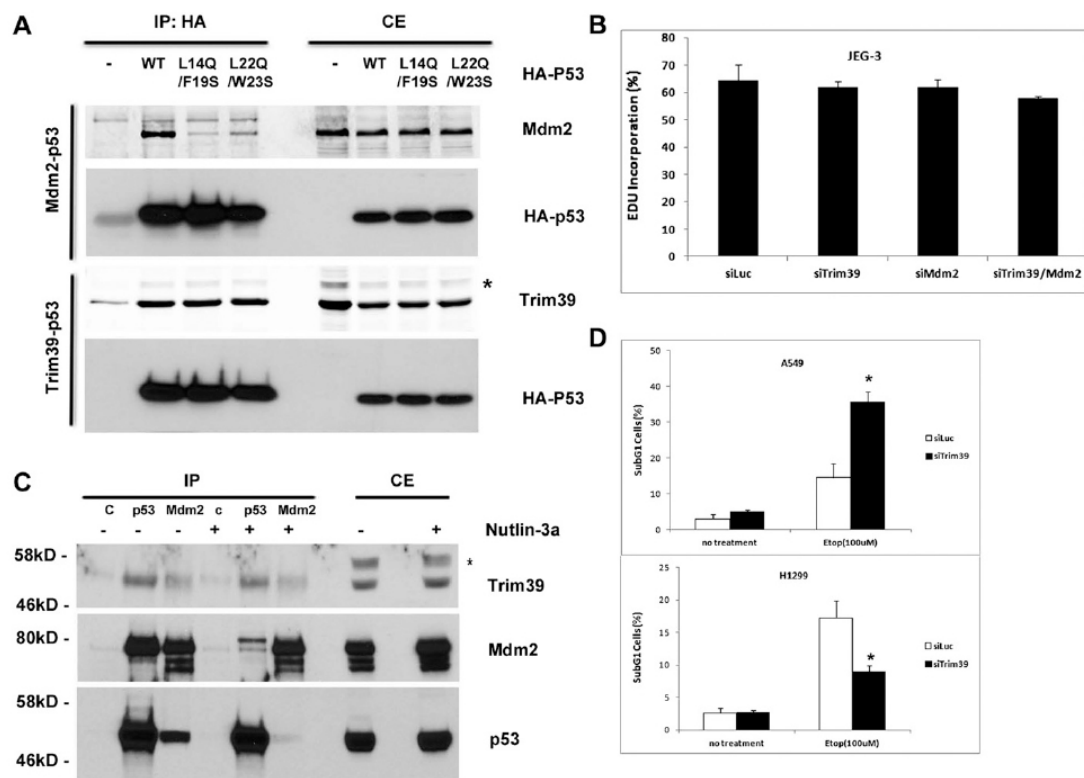


Figure 3.13: TRIM39 and MDM2 interact with p53 differently.

Figure 3.13: (A) 293T cells were transfected with HA-p53 and TRIM39 or MDM2 as indicated. Forty-eight hours later, cells were harvested and immunoprecipitation (IP) was carried out to detect the interaction between HA-p53 and TRIM39 or MDM2. CE, cell extract. *, nonspecific signal. (B) JEG-3 cells were transfected with control, TRIM39, or MDM2 siRNA, as indicated. Forty-eight hours later, cell proliferation was detected by EdU (5-ethynyl-2'-deoxyuridine) incorporation. (C) MCF7 cells were treated with nutlin-3a for 6 h, and endogenous p53 or MDM2 was immunoprecipitated from MCF7 cells. The interaction between TRIM39, p53, or MDM2 was detected by Western blotting. c, control. *, nonspecific signal. (D) A549 or H1299 cells were transfected with control or TRIM39 siRNA; 48 h later, cells were treated with 100 μ M etoposide for a further 48 h. Apoptosis (sub-G1 population) was detected by flow cytometry. *P < 0.05 vs. control.

4. Conclusion and perspectives

The coordination of cell cycle regulation and apoptosis signaling is critical for maintaining tissue homeostasis and differentiation. These controls are essential for determining whether a cell survives or dies when facing DNA damage or cellular stressors. If cell cycle and apoptosis controls are aberrant, detrimental outcomes such as cancer can occur. From past research, we know that these two pathways are complicated and integrated; therefore understanding the connection between these two pathways will further our understanding of how cells fine tune the two pathways and react in response to stress.

In the research described in this dissertation, we found that the TRIM39 protein regulates both the cell survival and cell death pathways via the APC/C and p53. We found that TRIM39, which is an E3 ubiquitin ligase, is a novel inhibitor of the APC/C, the master E3 ligase that marks critical cell cycle regulators for destruction. The identification of MOAP-1 as an APC/C substrate links APC/C function to apoptosis. Furthermore, the discovery that p53 can be ubiquitinated by TRIM39 demonstrates that the tumor suppressor or oncogenic role of TRIM39 must be cell context-dependent. Taken together, these findings suggest that inhibiting TRIM39 could potentially be a novel cancer treatment in particular cancer cell types.

4.1 TRIM39 is an inhibitor of APC/C

In chapter 2, we provide data demonstrating that TRIM39 can restrain APC/C activity. The TRIM39 project originated from the knowledge that there existed significant sequence similarity between TRIM39 and Xnf7, a *Xenopus* E3 ubiquitin ligase inhibitor of the APC/C. Like Xnf7, TRIM39 is a RING domain containing E3 ligase that can auto-ubiquitylate itself in vitro. We found that TRIM39 can inhibit the degradation of the canonical APC/C substrates Cyclin B and geminin in HeLa cell lysates, and this inhibition was not observed when lysates were treated with the TRIM39 RING domain mutant (C44A). The inhibition is directly mediated by the action of TRIM39 on APC/C because TRIM39 can inhibit APC/C targeted Cyclin B ubiquitylation in vitro.

Understanding the regulation of the APC/C is valuable for expanding our knowledge of cell cycle progression and other functions such as neuron growth where the APC/C is involved. TRIM39 provided a novel angle from which to investigate the mechanism of APC/C inhibition. From our work, we have found that the inhibition is not due to competition between the APC/C and TRIM39 for a common E2 ligase, because supplementation with excess UbcH10 (an APC/C E2) did not prevent TRIM39-mediated APC inhibition. Given that the RING domain is required for this inhibition and we know that TRIM39 can interact with subunits of the APC/C (data not shown), the observed inhibition could be the result of ubiquitylation of APC/C subunits by TRIM39, thereby preventing APC/C assembly or substrate recruitment. Interestingly,

preventing APC/C assembly has been reported as a mechanism of inhibition by the chicken anemia virus protein, Apoptin which inhibits APC/C formation by association with APC1 and release of Cdc27 and APC1 from the APC/C (Teodoro et al., 2004).

It is also possible that the E3 ligase activity of TRIM39 is required primarily for auto-ubiquitylation. In such a scenario, auto-ubiquitylated TRIM39 effectively competes with other APC/C substrates. In this regard, it is interesting that TRIM39 possesses two putative D-box motifs. We have found that TRIM39 auto-ubiquitylation may facilitate its inhibitory activity, since pre-ubiquitylating the TRIM39 C44A mutant with TRIM39 wild type protein can cause C44A protein inhibitory activity (data not shown). We are continuing to refine these findings by adjusting the pre-ubiquitylation time and protein levels. We have already received mass spectrometry data identifying the sites on TRIM39 modified by ubiquitylation. We will further determine whether TRIM39 mutant protein that is refractory to ubiquitylation loses its APC/C inhibitory ability.

Alternatively, TRIM39 may act like Emi1, which has been shown to interfere with poly-ubiquitin chain elongation (Wang and Kirschner, 2013). Similarly, our data demonstrate that Cyclin B is oligo-ubiquitylated instead of poly-ubiquitylated by APC/C in the presence of TRIM39, suggesting TRIM39 may alter the ubiquitin transfer efficiency from E2 to substrates of APC/C.

Mitotic cell death (or mitotic catastrophe) has been shown to kill transformed cells effectively. For example, microtubule inhibitors such as taxanes and the vinca alkaloids are used in breast, ovarian and lung cancer treatments (Zeng et al., 2010). These drugs induce SAC and inhibit the APC/C; therefore cells are arrested in prolonged mitosis and die. However, some cells can escape from SAC-enforced inhibition of the APC/C (mitotic slippage) and this slippage may reduce the cell death that occurs during prolonged mitosis. Moreover, these microtubule perturbation drugs have deleterious side effects because normal microtubule functions are required for vesicular trafficking and axonal transport (Chan et al., 2012). Therefore, finding a potential method to directly inhibit the APC/C without perturbing microtubules (perhaps by inducing TRIM39) might provide a means of targeting cancer cells. We have found TRIM39 can inhibit the APC/C in vitro and prevent degradation of Cyclin B and geminin in vivo. However, in the absence of DNA damage in p53-deficient cells, TRIM39 does not appear to have an overt cell cycle effect. (Note: the G1/S arrest in TRIM39 depleted cells results from the accumulation of p53). Thus, to activate TRIM39 ectopically will require either a simultaneous DNA damaging signal or in depth knowledge of the mechanism of activation.

Interestingly, a chemical inhibitor of the APC/C, tosyl-L-arginine methyl ester (TAME), was shown to affect the activity of APC/C^{Cdh1} only when Emi1 is ablated in cells (Zeng et al., 2010). Likewise, TRIM39 depletion may not have any effect due to masking

by other dominant inhibitors of the APC/C. Therefore, I propose to examine the cell cycle effects of TRIM39 by overexpressing TRIM39 proteins in Emi1 or Mad2/BubR1 depleted cells. Additionally, if we identify which regions of ubiquitylated TRIM39 potentiate its ability to inhibit the APC/C and which regions are required for binding/inhibiting the APC/C, we may be able to design a peptide contains the relevant ubiquitin modifications and can serve as a potent APC/C inhibitor. .

4.2 APC/C contributes to apoptotic signaling

In chapter 2, the data demonstrate that Cdh1 interacts with the Bax activator MOAP-1 through D-boxes on MOAP-1. This interaction triggers APC/C ubiquitylation of MOAP-1 and promotes MOAP-1 destruction. We also found that Cdh1 depletion enhanced MOAP-1 protein expression in cells, and consequently, Cdh1 depleted cells have a greater apoptotic response when treated with etoposide and cisplatin.

As discussed previously, APC/C^{Cdc20} can target the anti-apoptotic protein Mcl-1 after prolonged mitotic arrest to induce apoptosis (Harley et al., 2010). This apoptotic signal could be strengthened via MOAP-1 before MOAP-1 is degraded by APC/C^{Cdh1}. In addition, the APC/C^{Cdh1} substrate Cyclin B together with Cdk1 can mediate inhibitory phosphorylation on caspase-2, caspase- 8 and caspase-9 during mitosis, suggesting that the APC/C^{Cdh1} utilizes its substrates to fine tune the threshold of apoptosis during mitotic exit (Allan and Clarke, 2007) (Andersen et al., 2009) (Matthess et al., 2010).

Etoposide and cisplatin cause G2/M arrest and apoptosis in p53 mutant cells. We found that Cdh1 depletion enhances DNA damage drug induced apoptosis in 293T, PC3, and H1299 cells, as well as in a panel of p53 mutant cells (data not shown), and that cell death can be attenuated after MOAP-1 depletion. Interestingly, the viral proteins Apoptin and E4orf4 both cause apoptosis following G2/M arrest in p53 mutant cells (Kornitzer et al., 2001) (Teodoro et al., 2004) and APC/C inhibition leads to an increased cell death response in these p53 mutant cells overexpressing viral proteins. These studies suggest that inhibition of the APC/C sensitizes cells to virus induced apoptosis and raise the possibility of MOAP-1 involvement in this process.

4.3 TRIM39 is a negative regulator of p53

In chapter 3, we found that TRIM39 depletion causes G1/S arrest and reduced proliferation in RPE cells. We also determined that this arrest resulted from p21 induction after p53 stabilization. This increased amount of p53 is because of loss of the p53 E3 ligase TRIM39. We showed that TRIM39 can interact with p53 and promote its proteosomal degradation after polyubiquitylation. This function is, again, RING domain dependent. In contrast, this function is independent of APC/C inhibition by TRIM39 and is independent of the predominant p53 E3 ligase MDM2. We also demonstrated that the importance of TRIM39 is cell type dependent; for example, cell cycle arrest is more obvious in TRIM39 depleted RPE and A549 cells, as compared to MCF7 and 22Rv1 cells.

These findings suggest that various E3 ligases of p53 are differentially necessary for p53 regulation in specific tissues, specific conditions, and specific stresses.

These findings also help to explain the puzzle of why apoptosis is not always reduced after TRIM39 depletion in some cells where MOAP-1 protein level should already be decreased (chapter 2, data not shown). Interestingly, p53 is also required for the DNA damage induced-activation of APC/C in G2 (Wiebusch and Hagemeyer, 2010). The finding that the APC/C activates after DNA damage was initially confusing, because our data indicate that the APC/C should be weakened to maintain MOAP-1 expression in response to DNA damage. In p53 wildtype U2OS or HCT116 cells, DNA damage caused premature activation of the APC/C and led to the destruction of the APC/C substrate Plk1, an important mitotic kinase (Bassermann et al., 2008). These data suggest that DNA damage induced G2 arrest in p53 wildtype cells may be due to decreased Plk1 resulting from premature activation of the APC/C. This is quite interesting, since we observe that Plk1 inhibition by treating with either a Plk1 inhibitor or a siRNA targeting Plk1 can both reduce TRIM39 protein expression (Fig. 4.1A, 4.1B). Since decreased TRIM39 increases p53 protein, it will be interesting to see whether DNA damage initiated G2 arrest in p53 wildtype cells is augmented by TRIM39. On the other hand, cells without normal p53 will eventually bypass G2 arrest and enter into mitosis where TRIM39 levels can be maintained (since Plk1 is high in prometaphase) and can restrain APC/C activity for MOAP-1 destruction and trigger apoptosis once the damage is too

severe to be recoverable (Bunz et al., 1998; Giono and Manfredi, 2006). We are still trying to understand how TRIM39 is regulated by Plk1 and how TRIM39 affects p53 induction in G2 arrest phase after DNA damage. Moreover, we are working on elucidating whether apoptosis induced by Plk1 inhibition is TRIM39 dependent. This is important to investigate, because Plk1 inhibitors have been used for clinical trials, and understanding the involvement of TRIM39 could help to identify biomarkers that would aid in deciding whether a patient could benefit from Plk1 inhibitor treatment (Plyte and Musacchio, 2007) (Yim, 2013.) The proposed model is in Figure. 4.2.

Given down-regulation of p53 is common during cancer development, we postulated that high expression of TRIM39 might lead to p53 inactivation and enhanced tumor occurrence. We analyzed TRIM39 gene expression in cancer samples using cBioPortal database and we found the frequency of TRIM39 gene amplification is slightly higher in ovary, melanoma and liver cancers (Fig. 4.3) (Cerami et al., 2012) (Gao et al., 2013). At this stage, we do not know whether the protein is also overexpressed and might contribute to cancer development. However, the information from cBioPortal is suggestive; we will be interested in determining whether TRIM39 protein levels are higher in these types of cancer as well as whether p53 and TRIM39 protein levels are inversely correlated in these types of cancer. This research will help us gain more knowledge of the role of TRIM39 and may provide insights of value for cancer therapy.

4.4 The regulation of TRIM39

The E3 ligase TRIM39 can inhibit the APC/C, and this inhibition is essential for apoptosis induction upon DNA damage. The apoptosis proceeds via the release of cytochrome *c* from the mitochondria, which is controlled by Bcl-2 family proteins, particularly the pro-apoptotic protein Bax. The APC/C constitutively ubiquitylates the Bax-activator protein MOAP-1 and TRIM39 prevents this modification when cells are treated with DNA damaging reagents. We speculate that DNA damage response signaling is involved in the regulation of MOAP-1 stability, and this stability change is likely through altered TRIM39 activity. Indeed, we found that MOAP-1 protein is not elevated when cells are treated with Chk1 or ATR/M inhibitors one hour prior to etoposide treatment. In contrast, MOAP-1 protein expression is induced in cells treated with only etoposide, Chk2 inhibitor plus etoposide and Cdk1 inhibitor plus etoposide (Fig. 4.4A, 4.4B). Moreover, we found that TRIM39 ligase activity is increased by DNA damage, since we observed more ubiquitin conjugation on purified overexpressed or endogenous TRIM39 from etoposide treated lysates after incubating TRIM39 with E1, E2, ATP and ubiquitin (Fig. 4.5A, 4.5B). This increase in ligase activity is likely to explain the enhanced inhibitory activity toward the APC/C, since we have found that the inhibition requires TRIM39 ligase activity. We hypothesized that increased MOAP-1 levels resulted from increased TRIM39 ligase activity. Because the MOAP-1 level is changed when Chk1 is inhibited, we believe that the change stems from altered ligase

activity of TRIM39. As shown in Fig. 4.4C, ubiquitylation on TRIM39 is decreased in Chk1 depleted cells. These data are still preliminary as we don't know yet whether the ubiquitylation of TRIM39 in this setting is due to autoubiquitylation or ubiquitylation by another E3 ligase. We also need to further confirm whether Chk1 directly interacts with or phosphorylates TRIM39 to modulate its activity. It will be of interest to see how Chk1 signaling affects TRIM39 autoubiquitylation. MDM2 autoubiquitylation is reported to facilitate the interaction of E2-Ub and its substrates (Ranaweera and Yang, 2013). Therefore, DNA damage induced autoubiquitylation of TRIM39 may enhance its ability to inhibit the APC/C to stabilize MOAP-1.

TRIM39 isoforms can also auto-regulate TRIM39. As reported by another group also studying TRIM39, the half life of isoform 1 is short and the half life of isoform 1 is extended by associating with isoform 2 (used in our experiments). They also suggested that both isoforms of TRIM39 are required for preventing p21 ubiquitylation by CRL4^{Cdt2} in HCT116 cells (Zhang et al., 2012b). By forming a complex with p21, the two isoforms protect p21 from interacting with Cdt2. Intriguingly the p21 stabilization effect is neither E3 ligase dependent nor p53 dependent. In our hands, we observed p21 protein level is increased in TRIM39 depleted cells. We initially thought the discrepancy was due to a cell context dependent function of TRIM39 and we did not examine p21 levels in HCT116 cells at that time. However, we found that p21 is induced rather than decreased after TRIM39 depletion in RPE, MCF7, A549, 22Rv1 and HepG2 cells. Moreover, the

report is controversial since we observed less EdU incorporation after TRIM39 knock-down in HCT116, A4549, U-87MG, 22Rv1 and HepG2 cells whereas the group found opposite effect only in HCT116 cells. Therefore, whether p21 is really stabilized by interacting with TRIM39 still needs to be confirmed.

4.5 TRIM family modulates p53

We found that TRIM39 is a p53 ubiquitin E3 ligase. Interestingly, TRIM24 is also reported to target p53 for ubiquitylation and degradation (Allton et al., 2009). Besides the direct interaction with p53, there are other TRIM39 family proteins reported to modulate p53 stability. For example, PML (TRIM19) can sequester MDM2 to nucleolar caps to prevent p53 degradation once TRIM19 is phosphorylated by ATR after DNA damage (Bernardi et al., 2004). TRIM8 and TRIM13 both are reported to stabilize p53 by mediating MDM2 degradation (Caratozzolo et al., 2012) (Joo et al., 2011). Moreover, TRIM28 is reported to promote MDM2 mediated ubiquitylation on p53 by forming a complex with MAGE protein, which enhances MDM E3 ligase activity, interacting with MDM2 and decreasing acetylation of p53 (Doyle et al., 2010) (Wang et al., 2005). Besides affecting stability, TRIM29 is reported to repress the transcriptional activity of p53 by interacting with p53, promoting its nuclear export and by inhibiting its Lys120 site acetylation (Yuan et al., 2010b) (Yuan et al., 2010a).

TRIM proteins can form homodimers or heterodimers, which confer upon them a variety of substrate specificities or actions under different physiological conditions.

Therefore, these TRIM proteins could form heterodimers to regulate p53. Interestingly, TRIM39 can form a heterodimer with TRIM13 and TRIM13 protein is reported to promote MDM2 degradation. Therefore, I speculate that the degradation of p53 by TRIM39 will be counteracted by TRIM13 in cells that have both MDM2 and TRIM13 E3 ligase. It could also be possible that TRIM13 is sequestered by TRIM39, thereby preventing MDM2 degradation and promoting p53 destruction. In order to understand more physiological functions of TRIM39, it will be interesting to see how homodimers of TRIM39 and how heterodimers of TRIM39/13 influence TRIM39 E3 ligase activity and alter p53 stability (Horie-Inoue, 2013). Additionally, TRIM39 homodimerization may be important for its nuclear localization since its homologue Xnf7 has been reported to form homotrimers for enabling its chromosome association (Beenders et al., 2007). Thus, fully understanding the function and activity of TRIM39 may require the study of its oligomerization.

4.6 Concluding marks

The data presented in this dissertation have advanced our knowledge of how cell cycle and apoptosis are regulated by TRIM39 through the APC/C and p53. We found that TRIM39 is a RING domain E3 ligase that can autoubiquitylate and ubiquitylate substrates. We also demonstrate that TRIM39 is an inhibitor of the APC/C, and this inhibition prevents MOAP-1 destruction, which ultimately sensitizes p53 negative cells to chemotherapy drug induced apoptosis. In addition, we discovered that TRIM39 is a

ubiquitin E3 ligase for p53, which promotes the transcription of p21 and Bax. Therefore, TRIM39 expression and activity can modulate G1/S arrest and apoptosis.

Given the roles of the APC/C as a cell cycle gatekeeper essential for restricting cell division and p53 as a genome guardian critical for ensuring checkpoint or apoptosis response when facing stresses, understanding TRIM39 which controls both the APC/C and p53 may help to develop potential novel therapeutics for treating cancers and other diseases.

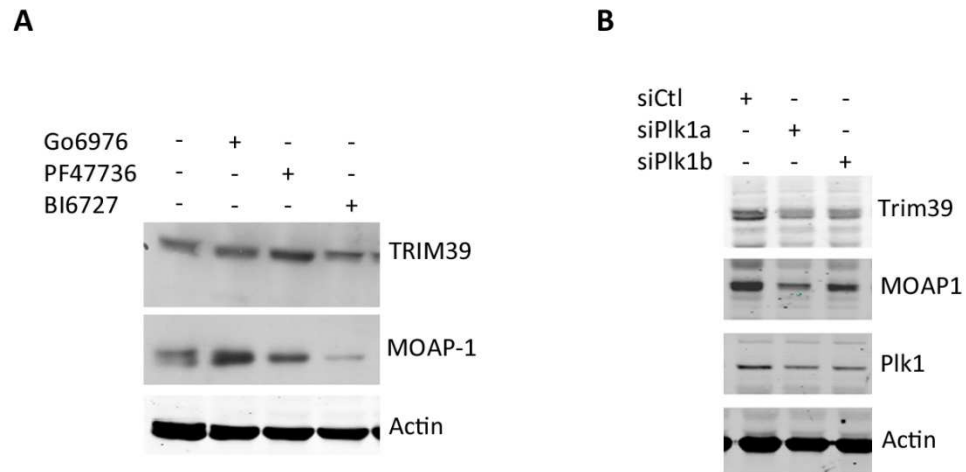


Figure 4. 1: Inhibition of Plk1 decreases TRIM39 amount.

Figure 4.1: (A) 293T cells were treated with 1 μ M Chk1 inhibitor Go6976, 0.4 μ M Chk1 inhibitor Pf47736 and 0.02 μ M Plk1 inhibitor BI6727 for 8 h then cells were collected and lysed. Cell lysates were analyzed by western blot and immunoblotted with indicated antibodies. (B) 293T cells were transfected with control (Ctl), Plk1 targeted siRNA (Plk1a and Plk1b) for 2 d. Cells were collected and lysed for analysis.

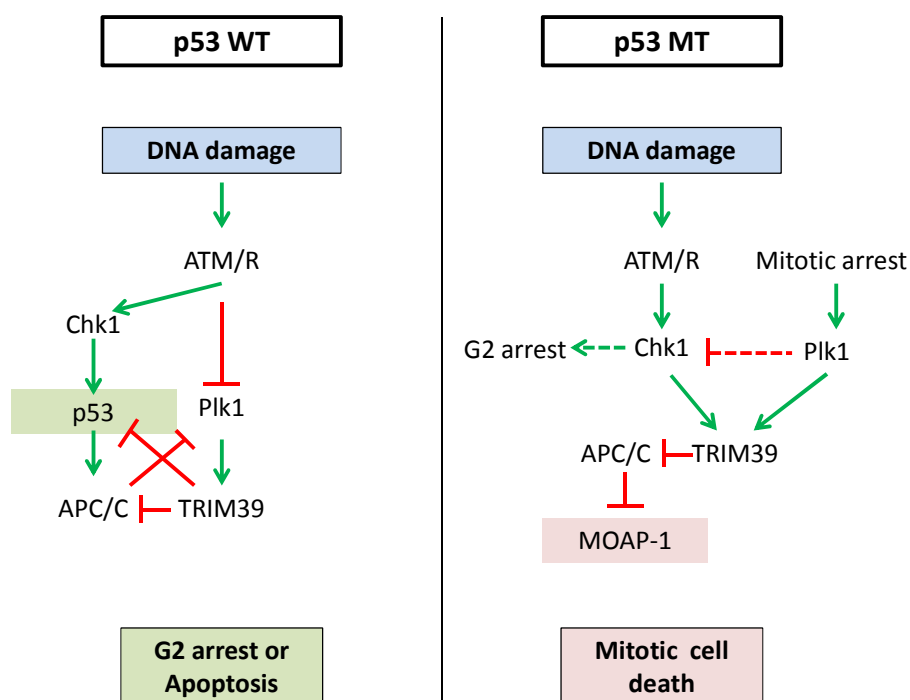


Figure 4. 2: Proposed model of TRIM39 signaling in p53 wildtype or mutant cells.

Figure 4.2: In p53 wildtype (WT) cells, DNA damage occurred after S phase will activate ATM/R which is followed by Chk1 activation and Plk1 inhibition. Chk1 activation leads to p53 accumulation which is required for sustained G2 arrest and the APC/C activation in G2. We also propose the inhibition of Plk1 will also decrease the amount of TRIM39, thereby preventing TRIM39 inhibition on the APC/C. p53 accumulation and the APC/C activation decreases the amount and activity of Cyclin B/Cdk complexes and arrests cells at G2 phase. Meanwhile, p53 will enhance apoptosis by upregulating pro-apoptotic genes. In some situations, p53 mutant cells will bypass G2 arrest and cells enter mitosis. At this stage, Plk1 may increase TRIM39 abundance and Chk1 may enhance TRIM39 ligase activity to inhibit the APC/C. Therefore, MOAP-1 is accumulated and stimulates apoptosis. (Green line: activation. Red line: inhibition. Dotted line: weak activity.)

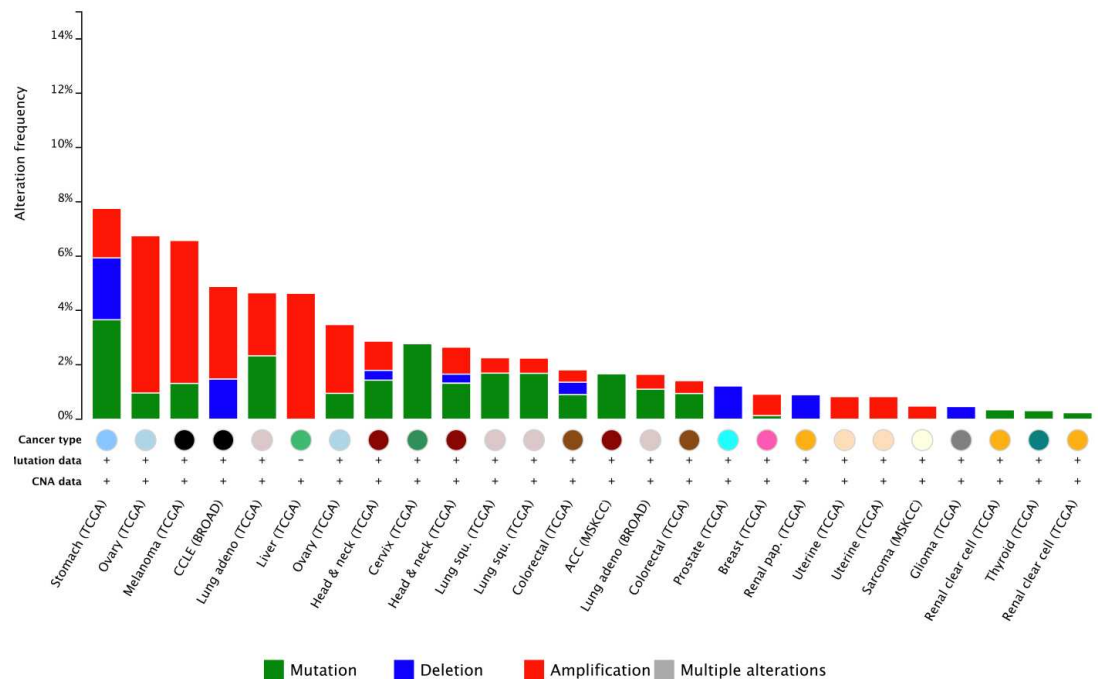


Figure 4. 3: Genomic alternation of TRIM39 analysis across various cancer types.

Figure 4.3: The figure is analyzed from cBioPortal database. X-axis represents various cancer types and Y-axis indicates the TRIM39 gene alteration frequency which is found in the samples collected by cBioPortal database. Amplification of TRIM39 (red rectangle) may correlate with the development of ovary, melanoma and liver cancers.

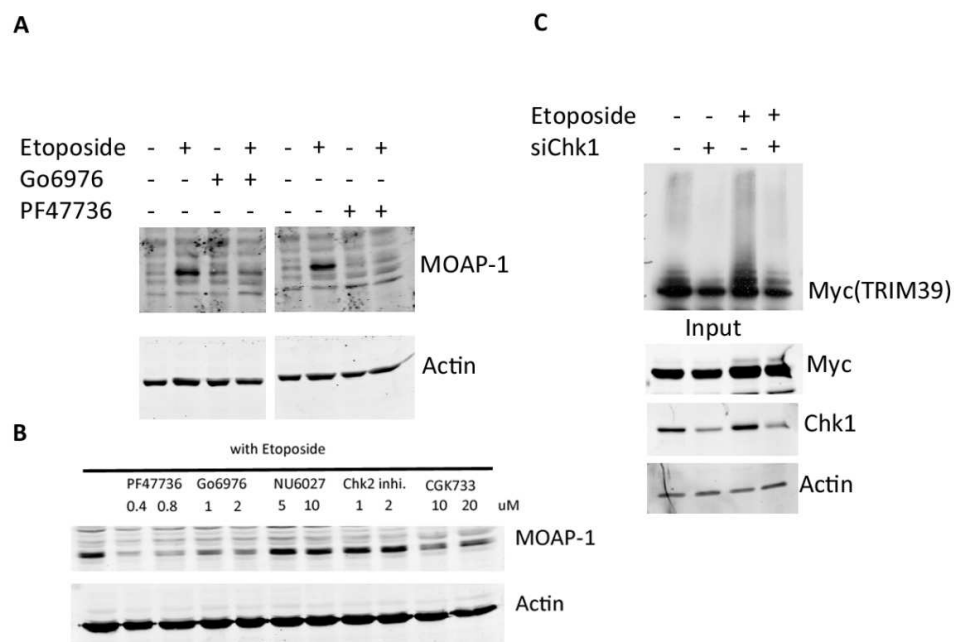


Figure 4. 4: Chk1 affects etoposide induced MOAP-1 stability and TRIM39 ubiquitylation.

Figure 4.4: (A) 293T Cells were treated with Chk1 inhibitor 1 μ M Go6976 and 0.4 μ M PF47736 one hour earlier than 100 μ M etoposide treatment. After 24 h, cells were collected and lysed for analysis. (B) 293T Cells were treated with chemical inhibitors as indicated concentration and 1 hour in advance than 100 μ M etoposide for 24 h. Cells were collected and lysed for immunoblotting. (C) 293T cells were transfected with control or Chk1 targeted siRNA. After 1 day, cells were transfected with Myc-TRIM39 plasmids and next day cells were treated with 100 μ M etoposide another day. Cell were lysed and immunoprecipitated with anti-Myc antibody.

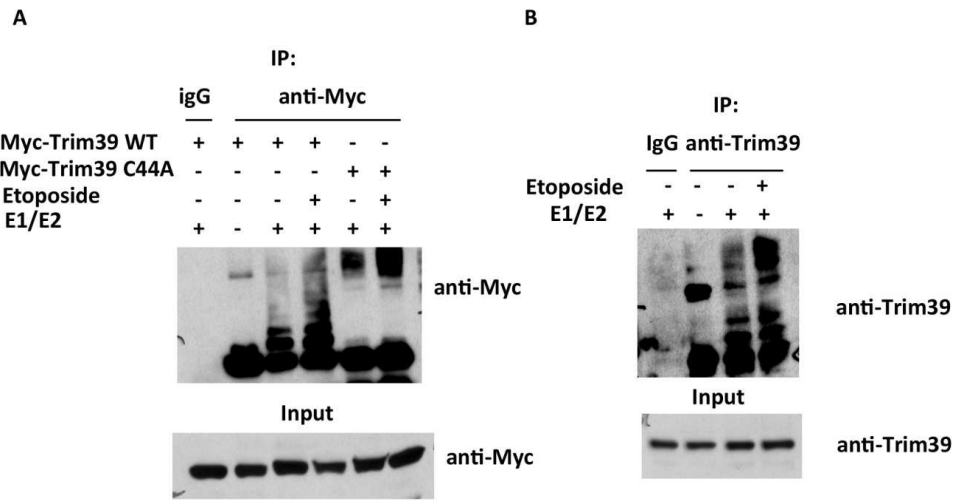


Figure 4. 5: DNA damage increases TRIM39 ligase activity

Figure 4.5: (A) 293T cells were transfected with 4 ug Myc-TRIM39 WT or C44A plasmid for 24 h and treated with 100 μ M etoposide for another 24 h before collecting. Cells were lysed and IP with Myc antibody for 6 h. Immunoprecipitated complexes were incubated with E1, E2, ATP, and Ub at 30 $^{\circ}$ C for 45 min. (B) 293T cells were treated with 100 μ M etoposide for 24 h. Cells lysates were made and IP was performed using TRIM39 antibody. Immunoprecipitated complex containing TRIM39 was used for in vitro ubiquitylation assay and samples were resolved in SDS-PAGE and examined by western blotting.

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Biography

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Education

Ph.D. in Pharmacology and Cancer Biology,
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Publications

1. Chen C, Zhang L, **Huang NJ**, Huang B, Kornbluth S, *PNAS* (2013), Suppression of DNA damage checkpoint signaling by Rsk-mediated phosphorylation of Mre11 (under revision)
2. Zhang L*, **Huang NJ***, Chen C, Tang W, Kornbluth S, *PNAS* (2012), 109(51):20931-6. Ubiquitylation of p53 by the APC/C inhibitor Trim39 (*: co-first authorship)
3. **Huang NJ***, Zhang L*, Tang W, Chen C and Kornbluth S, *J Cell Biol* (2012), 197(3):361-367. The Trim39 ubiquitin ligase inhibits APC/CCdh1-mediated degradation of the Bax activator MOAP-1(*: co-first authorship)
4. Chang CC, Naik MT, Huang YS, Jeng JC, Liao PH, Kuo HY, Ho CC, Hsieh YL, Lin CH, **Huang NJ**, Naik NM, Kung CC, Lin SY, Chen RH, Chang KS, Huang TH, Shih HM., *Mol Cell*. (2011), 42(1):62-74. Structural and functional roles of Daxx SIM phosphorylation in SUMO paralog-selective binding and apoptosis modulation.

Honors and Awards

1. **Fitzgerald scholars**, Department of Pharmacology and Cancer Biology, Duke University, 2012
2. **Hung Taiwan-Duke University Fellowship**, 2009 - 2011, Institute of Biomedical Sciences, Academia Sinica and Graduate School, Duke University
3. **Selective Award of annual student poster competition**, Institute of Biochemical Science at National Taiwan University, 2008
4. **Alumni Association scholarship**, Department of Agronomy at National Taiwan University, 2005
5. **National Taiwan University Presidential Award**, 2005, 2004, 2002